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(71) Applicant: UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; Chapel Hill, NC 27599-4100 (US).

(72) Inventors: LEMON, Stanley, M. ; 101 Pine Lane, Chapel Hill, NC 27514 (US). JANSEN, Robert, W. ; 1A Mannsfield Condominiums, Pittsboro, NC 27312 (US).

(74) Agent: COOPER, Iver, P.; Browdy and Neimark, 419 Seventh St., Ste. 300, Washington, DC 20004 (US).

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(54) Title: IMMUNOLOGICAL DOMAINS OF HEPATITIS DELTA VIRUS ANTIGEN

(57) Abstract

In an effort to map the antigenic domains of HDAg, 209 overlapping hexapeptides, spanning the entire 214 amino acid residues of the protein, were synthesized on polyethylene pins and probed by ELISA with sera containing high titers of anti-HD antibodies. Domains recognized by antibodies present in serum from human chronic carriers of this virus included residues 2-7, 63-74, 86-91, 94-100, 159-172, 174-195 and 197-207. Oligopeptides 15 to 29 residues in length and representing epitopes of HDAg found to be dominant in man (residues 2-17, 156-184 and 197-211) were synthesized in bulk and found to possess significant antigenic activity by microtiter ELISA. The reacativity of the 197-211 peptide with human sera confirms that the entire 214 amino acids of HDAg are expressed during infection in vivo. The aforementioned peptides are useful as diagnostic reagents and as vaccines.

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IMMUNOLOGICAL DOMAINS OF HEPATITIS DELTA VIRUS ANTIGEN

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to synthetic peptides useful as diagnostic reagents in the detection of Hepatitis delta virus infection, as well as, in the form of an immunogenic conjugate, as vaccines.

Information Disclosure Statement

Hepatitis delta virus (HDV) is a unique human pathogen which has been implicated, in association with hepatitis B virus (HBV), as a cause of severe acute hepatitis and progressive chronic liver disease (7). The HDV virion consists of a circular, single-stranded RNA genome approximately 1.7 kilobases in length (17,19,26,27) and a highly basic phosphoprotein (hepatitis delta antigen, or HDAg) (4,5) packaged within an envelope comprised of hepatitis B surface antigen (HBsAg) (3,24). HBsAg present in the HDV virion is encoded by HBV DNA, and current data indicate that HBV confection is an absolute prerequisite for significant infection and disease associated with HDV in man.

Complete genomic sequences have been independently derived from cDNA clones established from two different HDV strains, one after chimpanzee passage (26,27) and the other directly from human serum (19). Analysis of these sequences has shown that virion RNA contains 2 open reading frames capable of encoding proteins of longer than 100 amino acids, while the antigenomic sequence contains 3 additional open reading frames of equivalent length (19,26,27). Thus, the HDV genome potentially encodes up to 5 different proteins of significant length. HDAg has been shown to be encoded by one

of the open reading frames in antigenomic sense RNA (5,28): ORF 5 according to the nomenclature of Wang et al. (26,27), or ORF-2 according to Makino et al. (19). Thus, HDV is a negative-stranded RNA virus. HDAg is present in HDV particles in two distinct forms having molecular weights of 27,000 (p27d) and 24,000 daltons (p24d) (3,28). Present knowledge of the differences between these two HDAg species is incomplete. ORF 5 potentially encodes a protein 214 amino acids in length, thought to represent p27d (19,26,27). It has been suggested that the p24d form of HDAg may represent expression of a C-terminal truncated protein of 195 amino acids, due to heterogeneity in the HDV genome reflected by the presence of an amber stop codon in some cloned cDNAs (28). Alternatively, the two molecular species may reflect posttranslational processing of the primary ORF 5 expression product. HDAg is phosphorylated at multiple serine residues, and has been shown to have RNA-binding activity of uncertain specificity (5). Thus far, it is the only protein product clearly recognized to be expressed by the HDV genome during in vivo infection. Further understanding of the structure and function of this viral protein is central to unraveling the molecular events involved in HDV replication and the pathogenesis of delta hepatitis.

Houghton, EP Appl. 251,575 sequenced ORF5 and set forth a predicted amino acid sequence for the encoded protein. They suggested that this protein, or antigenic fragments thereof, might be useful for HDV diagnosis and vaccination. However, no epitope mapping was in fact performed and Houghton provides no guidance as to which subsequences of ORF5 might be antigenic determinants of HDV.

One traditional method of mapping the antigenic determinants (epitopes) of protein antigens was to laboriously prepare a large number of well-characterized chemical derivatives and peptide fragments from the original protein and then to test the derivatives for immunological activity.

Developments in the art of solid state peptide synthesis have provided an alternative approach to obtaining potentially antigenic subsequences of a known protein antigen. By relaxing the usual criteria for quantity and purity, a large set of overlapping peptides corresponding to a larger protein may be prepared (11-12,29).

An important limitation of the pin-based oligopeptide approach to epitope mapping is related to the purity of the peptides synthesized on pins. As these peptides are not cleaved from the pins following their synthesis and undergo no physical purification steps, impurities due to unplanned side chain reactions or other irregularities in the synthesis accumulate with the addition of residues to the immobilized peptide. Thus, there are inherent limitations to the length of peptides that can be usefully synthesized in this fashion. Indeed, its developers have remarked that "because of the uncertainty of the final purity of the peptide on any given rod, the methods suffers from the disadvantage that a negative result cannot be taken as proof of the absence of antibody able to bind that nominal sequence." They also emphasize the importance of highly selective antibodies, preferably monoclonal antibodies, for screening purposes. Monoclonal antibodies against HDV delta antigen are not yet commercially available.

It should also be considered that if hexapeptides are prepared, so as to minimize the purity problem, epitopes may be overlooked since 6-mers are the smallest peptides that would be expected to have a reasonable probability of preserving antigenic activity (12). Even so, a hexapeptide in isolation will not necessarily assume the exact same conformation that it does when it is a subsequence of a protein, so its affinity for antibodies may be different than that of the corresponding site on the protein.

Finally, while the assay identifies potential epitopes, it tells nothing about their uniqueness.

A number of mathematical methods have been developed for using amino acid sequence data to locate protein determinants. These look, for example, at local average hydrophilicity (14). These methods have their limitations, however. Hopp (14), for example, was only confident of the antigenicity of his peak hydrophilic peptide; the second and third highest peaks resulted in a mixture of correct and incorrect assignments. Garnier (10), reviewing Chou and Fasman's (6) method of predicting the secondary structure, found that it was only about 60% accurate in assigning residues to one of four conformational states. These deficiencies are attributable partially to the relatively small size of the database used to parametrize the models, and partially to the fact that the assumption that these characteristics are purely locally determined is simplistic.

SUMMARY OF THE INVENTION

Surprisingly, we have found that the major antigenic domains of the human hepatitis delta virus antigen which are recognized by human antibodies are found in the less hydrophilic carboxyl region (residues 145-214) of the protein. In particular, the domains in the region 159-207 appear dominant.

These domains may be used in the form of synthetic oligopeptides as diagnostic reagents. Many different assay formats exist, and the oligopeptides may be labeled or immobilized as required by the format.

The oligopeptides may also be conjugated to an immunogenic carrier and used to elicit an HDV-specific immune response to the oligopeptide. The monoclonal or polyclonal

antibodies produced after such immunization may likewise be used as diagnostic reagents. Additionally, the immunogenic conjugates may be used as vaccines.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Analysis of the predicted HDAg (19) secondary structure by the PEPTIDESTRUCTURE program (15). The graphical output of the PLOTSTRUCTURE program, detailing hydrophilicity (Kyte-Doolittle), surface probability (Emini), chain flexibility (Karplus-Schulz), antigenic index (Jameson-Wolf), and secondary structure by methods of Chou-Fasman (CF) and Garnier-Osguthorpe-Robson (GOR) is shown.

Figure 2: Amino acid sequences of several screened HDV peptides. Note that the leading cysteines are provided for coupling purposes and are not a part of the cognate HDV sequences. Their presence is believed to be optional, as indicated in the claims by parenthesizing them.

Figure 3: shows the results of screening six oligopeptides for binding by 23 anti-Hd positive sera and 17 antgi-HD negative sera. All sera wsere HBsAg positive. The oligopeptides corresponded to HDAg 2-17, 58-78, 82-102, 123-143, 156-184 and 197-211.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The approach we utilized for mapping the epitopes of HDAg involved the synthesis of 209 hexapeptides, overlapping each other by five residues and spanning the entire 214 residues that most likely comprise the p27d form of HDAg. These peptides were synthesized on polyethylene pins configured in a microtiter format, a method first described by Geysen et al. (11) that allows their direct and repeated use in ELISA tests to determine antibody binding activity.

The peptides of the present invention may be prepared in any convenient manner. Preferably, they are prepared by purely chemical synthesis. For direct chemical synthesis of peptides, see Merrifield (21). Secondly, they may be prepared by expression of the peptides or of a larger peptide including the desired peptide from a corresponding gene (whether synthetic or natural in origin) in a suitable host. These techniques may be combined. This fusion peptide may contain a cleavage site whereby the peptide of interest may be released by cleavage of the fused molecule.

Preferably, the peptide comprises a sequence substantially homologous with one of the domains of Table 2; more preferably, it is one of the peptides defined in Figure 2. However, analogous peptides with similar immunological activity are encompassed by the present invention. These peptidies may differ from those recited by one or more substitutions, insertions or deletions of amino acids, either within the peptides or at the termini. Preferred substitutions are indicated by the table below of groups of often equivalent amino acids (with single letter codes indicated in parenthesis):

(a) Ala(A) Ser(S) Thr(T) Pro(P) Gly(G);
(b) Asn(N) Asp(D) Glu(E) Gln(Q);
(c) His(H) Arg(R) Lys(K);
(d) Met(M) Leu(L) Ile(I) Val(V);
(e) Phe(F) Tyr(Y) Trp(W); and
(f) Cys(C).

Candidate analogues may be chosen on the basis of the effect of the amino acid changes on local hydrophilicity, surface probability, flexibility, antigenic index and secondary structure, as well as on the immunoreactivity of the most homologous hexapeptides. The peptides used as reagents or vaccines preferably include at least ten amino acids of an antigenic domain of HDAg, and more preferably at least twenty amino acides. Smaller peptides are less likely to accurately

reproduce the desired antigenic domain in its native conformation. They preferably should not exceed 50 amino acids, and more preferably 30 amino acids, in length. This upper limit is partially a function of synthetic considerations. Additionally, it may be desirable that the peptide mimic only selected epitopes of HFAg, since some epitopes will be less cross-reactive with epitopes of non-HFAg protein than others.

The peptide may also comprise a repeated sequence corresponding to one or more of the antigenic domains identified herein, with or without a spacer region separating the repeats.

In one embodiment, a panel of peptides according to the present invention are used as diagnostic agents. As will be seen hereafter, no one peptide determinant was recognized by all of the delta positive sera considered. However, a panel comprising a small number of oligopeptides, each corresponding to a significant antigenic domain of delta antigen, is more readily prepared and characterized than is a diagnostic reagent comprising the entire 200+ amino acid delta antigen.

A peptide of particular interest, though not listed in Figure 1, is 156 (or 159) to 195 (or 212).

The peptides may be conjugated to an immunogenic carrier, which may be, e.g., a polypeptide or polysaccharide. If the carrier is a polypeptide, the desired conjugate may be expressed as a fusion protein. Alternatively, the HDV-specific peptide and the carrier may be obtained separately and then conjugated.

Numerous enzyme immunoassay formats, labels, conjugation and immobilization techniques, etc., are disclosed in the following publications, hereby incorporated by reference herein: O'Sullivan, Annals Clin. Biochem., 16:221-240 (1976);

McLaren, Med. Lab. Sci., 38:245-51 (1981); Ollerich, J. Clin. Chem. Clin. Biochem., 22:895-904 (1984); Ngo and Lenhoff, Mol. Cell. Biochem., 44:3-12 (1982). The disclosed peptides may be labeled or immobilized, directly or indirectly, as required by the format selected. The present invention is directed generally to the use of the disclosed haptens and antigens in assays for HDV antigens and antibodies in body fluids of infected subjects and is not limited to any particular form of immunoassay.

The label may be, without limitation, an enzyme, enzyme substrate, radioisotope, or fluorescent label and may be attached directly or via an antibody-antigen, carbohydrate-lectin or biotin-avidin bridge. If immobilized, covalent or noncovalent means may be used to associate the peptide with the desired support, which may be for example a plate, tube, dipstick, bead or particle.

The invention in certain of its preferred embodiments is further described in the Examples which follow.

Materials and Methods

Human sera

Sera were collected from hemophiliac patients who were chronic carriers of HBV (HBsAg-positive) and enrolled in a longitudinal study exploring the contribution of HDV infection to chronic liver disease in hemophilia. Sera with uniquely high anti-HD antibody titers were chosen for these studies to enhance the signal to noise ratio in epitope screening by hexapeptide ELISA. These sera represented a select group (less than 10%) of all anti-HD positive human sera. Preferably, the anti-HD titer (measured as for Table 1) is at least about 1:12,500 (cp. AF043). Sera having lesser titers of anti-HD antibody provided no useful information in hexapeptide ELISA assays. Control sera were obtained from two healthy

individuals of comparable age and sex, one of whom was an anti-HD negative, chronic HBsAg carrier. Additional sera were collected from a woodchuck (Marmota monax), WC862, which was positive for woodchuck hepatitis virus (WHV) and experimentally superinfected with human HDV. WHV is an hepadnavirus that is closely related to HBV and capable of supplying helper functions necessary for the replication of HDV (22). The anti-HD activity of these sera was determined by a commercially available competitive ELISA (Delta EIA, Abbott Laboratories, N. Chicago, IL). HBsAg was also detected in sera by a commercial ELISA (Auszyme, Abbott Laboratories).

Anti-HD radioimmunoassay

Additional testing for anti-HD was carried out by a microtiter solid-phase competitive radioimmunoassay modified from that described by Rizzetto et al. (25). HDAg employed in this assay was extracted from the liver of an acutely superinfected, WHV-positive woodchuck (WC643) by preparing tissue homogenates in 6 M guanidine HCl (pH 6.0), followed by dialysis against phosphate buffered saline (PBS), as described by Bergmann and Gerin (1). Flexible polyvinyl chloride microtiter plate wells were coated for 2 hr at 35°C with 100 µl of a human anti-HD positive serum (AI035) diluted 1:4000 in 50 mM carbonate buffer, pH 9.6, washed with PBS containing 0.05% Tween 20 (PBS-T), and loaded with 40 µl of the HDAg preparation. After 2 h incubation at 37°C, the plates were washed with PBS-T, and 50 µl of a 1:100 dilution of test serum in PBS was added to each well. Following an overnight incubation at 4°C, wells were washed with PBS-T and incubated for 4 hr at 4°C with 50 µl of [125I]-labelled IgG (50,000 cpm), isolated from the serum of an anti-HD positive hemophiliac patient (ET87) by chromatography through DEAE-Sephacryl (Pharmacia, Piscataway NJ), iodinated by the chloramine-T method, and diluted in 10% fetal calf serum.

Immunoblot detection of anti-HD

Immunoblots were carried out with HDAg prepared from woodchuck serum, as this source provides antigen of less complexity than that present in liver (1). Serum (1.0 ml) taken from an acutely superinfected woodchuck (WC643) was layered over an 11 ml cushion containing 20% sucrose in 0.02 M HEPES (pH 7.4), 0.01 M $CaCl_2$, 0.01 M $MgCl_2$ and 0.1% bovine serum albumin, and centrifuged for 5 hr at 150,000 x G in an SW40 rotor (Beckman, Palo Alto CA). Pelleted HDAg was resuspended in distilled water and stored at -70°C until use. Antigen diluted in sample buffer (0.0625 M Tris, pH 6.8, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue) was separated by SDS-PAGE with stacking and separating gels containing 4% and 12.5% polyacrylamide respectively. Separated polypeptides were electrophoretically transferred to nitrocellulose paper at 85 mA for 3 hr at 4°C. Nitrocellulose papers were blocked with blocking buffer (3% milk, 50mM Tris, 150mM NaCl, 5mM EDTA, 0.25% gelatin, 0.05% NaN_3, pH 7.4) for 30 min, and incubated with test serum diluted 1:1000 in blocking buffer for 1 hr at room temperature. After washing in PBS-T, nitrocellulose papers were incubated for 30 min with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Bethesda Research Laboratories, Gaithersberg, MD) diluted in PBS. Following an additional washing step, the papers were placed in freshly prepared substrate solution (25 mg 3,3-diaminobenzidine in 50 ml 0.05 M Tris, 0.02% hydrogen peroxide, pH 7.4) for color development.

Pin-based oligopeptide synthesis

Overlapping hexapeptides spanning the entire HDAg molecule were synthesized on polyethylene pins with materials provided as components of the "Epitope Mapping Kit" manufactured by Cambridge Research Biochemicals, Inc. (Valley Stream, NY). This method of peptide synthesis employs

preformed active ester coupling with 9-fluoroenylmethyl-oxycarbonyl (FMOC) and t-butyloxycarbonyl (TBOC) protection. Its application to epitope mapping has been extensively described by Geysen et al. (11-13).

Pin-based oligopeptide ELISA

Oligopeptide-bearing pins, after sonication as described below, were blocked by incubation for 1 hr in microtiter plates containing 200 ul per well blocking buffer at room temperature. Pins were then transferred to a microtiter plate containing 175 ul per well of test serum diluted 1:6000 or more in blocking buffer. Following incubation at 4°C for 24 to 48 hr, pins were subjected to 3 cycles of washing in PBS-T, 30 min per cycle with agitation, and transferred to wells containing HRP-conjugated goat anti-human IgG diluted in blocking buffer without sodium azide. Woodchuck antibody was detected by sequential incubations with rabbit antiserum raised to woodchuck immunoglobulin, and HRP-conjugated goat anti-rabbit IgG. After washing as above, pins were placed in microtiter wells containing 150 μl of freshly prepared substrate solution (25 mg 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (ABTS, Boehringer-Mannheim Biochemicals, Indianapolis, IN) in 50 ml of 0.1 M disodium hydrogen phosphate, 0.08 M citric acid, 0.015% hydrogen peroxide, pH 4.0) and held at room temperature in the dark for 30 min. Color development was stopped by removing the pins and the absorbance of the substrate solutions determined immediately by reading in an automated ELISA plate reader at 405 nm. Data were downloaded to an IBM-XT computer and analyzed using Lotus 1-2-3 software. Reactions with human sera were considered positive if absorbance was > 0.2, while woodchuck serum reactions were considered positive if absorbance was > 0.5. Pins were stripped of immunoglobulin by sonication for 30 min

in 1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol and 0.1 M sodium dihydrogen orthophosphate at 60°C, washed with hot (60°C) distilled water followed by boiling methanol for 2 min, and air dried.

Bulk synthesis of oligopeptides

Peptide synthesis was carried out with a Biosearch Model 9500 Peptide Synthesizer (Novato CA). TBOC chemistry was employed for peptide synthesis (21).

Oligopeptide ELISA for anti-HD

Oligopeptides (750 ng in 75 µl carbonate buffer) were applied to the wells of flat-bottom ELISA plates (Falcon) by incubation for 2 hours at 35°C in a humidified chamber. Plates were washed with PBS-T and blocked by the addition of 75 µl blocking buffer. Serum specimens diluted in blocking buffer (50 µl per well) were added to the plates which were then incubated overnight at 4°C. Following washing with PBS-T, 50 µl of HRP-conjugated goat anti-human IgG diluted in blocking buffer without sodium azide was added for 50 min at 37°C. Plates were washed with PBS, followed by the addition of o-phenylenediamine substrate solution (55 µl per well) at room temperature for 15 min in the dark. Color development was stopped by the addition of 160 µl of 1 N H_2SO4, and the absorbance at 490 nm determined by reading in an automated ELISA plate reader.

HDAg secondary structure predictions

Hydrophilic domains of HDAg were predicted by the method of Hopp and Woods (14), while hydrophobic regions were independently predicted by a program which determined the mean hydrophobicity score for 11 residue windows using hydrophobic indices derived for individual side chains by Fauchere and Pliska (9). More detailed predictions of the secondary

structure of HDAg were done by means of the PEPTIDESTRUCTURE and PLOTSTRUCTURE programs of the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (15). These programs include predictions of hydrophilicity according to the method of Kyte and Doolittle (18), peptide secondary structure according to Chou and Fasman (6) and Garnier et al. (10), and surface probability according to Emini et al. (8). From these predictive measures, an antigenic index (AI) is derived by the algorithm developed by Jameson and Wolf (15). The AI is a measure of the probability that a domain is antigenic, and is calculated by summing weighted values for surface accessibility, regional backbone flexibility, and certain features of predicted secondary structure. In addition, regions predicted to be amphipathic alpha helices (putative T cell determinants) were identified by a program developed by Margalit et al. (20).

Results

HDAg epitope mapping with overlapping hexapeptides

We synthesized 209 overlapping hexapeptides spanning the 214 amino acids of human HDAg (ORF-2 expression product) predicted from the nucleotide sequence of cloned HDV cDNA reported by Makino et al. (19). Hexapeptides were synthesized on polyethylene pins using a synthesis system supplied by Cambridge Research Biochemicals. Control peptides (4-mers) included in the synthesis reacted specifically with murine monoclonal antibody supplied by the manufacturer in subsequent pin-based ELISA tests (mean signal to noise (S/N) ratio was 4), confirming the validity of the synthesis. However, preliminary pin-based ELISA tests demonstrated substantial non-specific binding of human IgG to pins supporting HDAg hexapeptides at test serum dilutions below 1:2000.

To overcome this problem, we determined the endpoint titer of each of a panel of 27 anti-HD positive sera in a competitive radioimmunoassay which employed as antigen HDAg extracted from woodchuck liver. These sera had been collected from hemophiliac patients who were chronic carriers of HBsAg. We selected for further study 5 sera with particularly high titers of anti-HD, ranging from 1:12,500 to > 1:312,500 (Table 1). These titers of anti-HD are highly suggestive of chronic infection with HDV. Each of these 5 sera were strongly positive in immunoblot assays against denatured HDAg concentrated from woodchuck serum, reacting with both p24d and p27d (Figure 1). We thus considered it likely that these sera had high titer antibody against sequential epitopes of HDAg that would be detectable in hexapeptide ELISA assays. Each serum was tested at a dilution of 1:6000 to 1:8000 against the array of HDAg hexapeptides.

Antibodies present in this serum (AC036) demonstrated binding activity against hexapeptides representing at least six discrete regions in the linear sequence of the HDAg protein. Epitopes recognized by binding of antibody to hexapeptides spanned residues 2-7, 63-69, 159-165, 167-172, 174-181, and 201-207. Additional hexapeptides demonstrating low level binding (above background, but less than the arbitrary cut-off of absorbance of > 0.2) represented residues 85-91, and 152-157 (Figure 2). These hexapeptides may represent minor determinants. Hexapeptide ELISA results with this positive serum were highly reproducible, while two anti-HD negative sera (A008 and A073, Table 1) yielded consistently negative results (mean OD490 0.113 and 0.121, and maximum OD490 0.176 and 0.157, respectively) when tested against the hexapeptide-bearing pins at similar dilutions (results not shown). Results of assays with alternating positive and negative sera demonstrated that the stripping procedure was effective in completely removing IgG bound to the pins. In the course of these studies, the hexapeptide-bearing pins were reused through 26 repetitive ELISA cycles without noticeable loss of activity.

Subsequent screening of HDAg hexapeptides with four other high-titer anti-HD positive human sera identified identical or closely positioned epitopes within the protein, as well as two additional antigenic domains. Antigenic domains of HDAg were defined as regions containing overlapping or contiguous hexapeptides found to be antigenic in screening with any of the 5 human anti-HD positive sera. Altogether, these domains included regions spanned by residues 2-7, 63-74, 86-91, 94-100, 159-172, 174-195, and 197-207 (Table 2). Although the carboxyl terminal 50 residues of the HDAg protein (residues 159-207) appeared immunodominant with each of the human sera tested in these assays, significant variation was evident between individual sera with respect to the degree to which certain hexapeptides were bound by antibody. Among the 5 anti-HD sera tested, AD099 demonstrated relatively low level binding of antibodies to HDAg hexapeptides (maximum OD490 of 0.398, compared with 0.858-2.272 obtained with the other four sera). These results suggest that the sequential epitopes recognized by AD099 antibodies in HDAg immunoblots may be poorly mimicked by synthetic peptides as short as six residues in length.

Mapping of HDAg epitopes recognized by the woodchuck

Hexapeptide ELISA tests were carried out with serum from a WHV-carrier woodchuck (WC862) which was acutely superinfected with HDV. Serum was collected following HDAg clearance from serum and the appearance of anti-HD. The second antibody employed in these hexapeptide ELISAs, rabbit anti-woodchuck immunoglobulin, generated higher background activity and required a different standard for positivity (OD490 > 0.5). The results of these tests indicated that the epitopes bound by woodchuck antibodies (residues 1-7, 63-71, 121-128, and 197-204) overlap with at least some of the antigenic domains recognized by humans (residues 2-7, 63-74, and 197-207). However, in contrast to the apparent immunodominance of the carboxyl terminal region of HDAg in humans, HDAg domains

defined by hexapeptides spanning residues 1-7 and 63-71 were dominant in this one woodchuck. In addition, woodchuck antibodies bound also to hexapeptides spanning residues 121-128, a region not recognized by any of the human sera tested (Table 2). These results suggest significant inter-species differences in the recognition of HDAg epitopes.

Comparison of hexapeptide ELISA with computer algorithm predictions

We analyzed the predicted amino acid sequence of HDAg for regions of relative hydrophilicity and hydrophobicity using the method of Hopp and Woods (14) and values of relative hydrophobicity developed for individual amino acid side chains by Fauchere and Pliska (9). Although these two methods yield somewhat different predictions of the hydrophilic and hydrophobic domains of the protein, both suggest the carboxyl terminal region (residues 145-214) of HDAg is less hydrophilic than the amino terminal 144 residues (not shown). Neither analysis would have predicted the antigenic domains occurring within residues 159-207 that appear dominant in hexapeptide ELISAs with human sera.

Predictions of the secondary structure of HDAg made by the PEPTIDESTRUCTURE program (15) are shown in Figure 6.

To determine the extent of correlation between residues predicted to be antigenic by this program and those observed to part of antigenic domains in hexapeptide ELISA assays, a statistical analysis was carried out. For each residue within the protein, the observed antigenic activity (i.e., inclusion within an antigenic domain) was cross-classified against predicted antigenic activity derived by each of the five separate algorithms contained within PEPTIDE STRUCTURE (15). The prediction criteria chosen for this analysis included (a) hydrophilicity score (18) greater than or equal to 1.3, (b) surface probability score (8) greater than or

equal to 5, (c) flexibility score greater than or equal to 1.04, (d) antigenic index (15) greater than or equal to 1.2, and the presence of a turn ("T" or "t") predicted by Chou-Fasman (6) or method of Garnier (10). Each prediction method was found to have relatively poor sensitivity or specificity (Table 7). Regardless of the prediction rule, the kappa statistic, which measures chance-adjusted agreement, never exceed 0.21, indicating poor agreement between the methods.

Finally, although regions predicted to be amphipathic alpha helices are generally considered predictive of T cell and not B cell determinants (20), it was of interest that 5 of 9 predicted amphipathic segments (residues 117-120, 131-133, 150-159, 170-177 and 179-183) were adjacent to or overlapped with antigenic sites determined in hexapeptide ELISAs.

Microtiter oligopeptide ELISA for anti-HD

Based on the results of hexapeptide ELISA tests with human sera, four oligopeptides representing HDAg residues 2-17, 156-184, 167-184, and 197-211 were synthesized in bulk and tested for antigenic activity by a microtiter ELISA. Maximal antigenic activity was associated with the largest peptide, representing residues 156-184, which demonstrated substantial antigenic activity with as little as 75 pg of peptide applied in solution to ELISA plate wells. The 156-184 peptide contains an internal tri-peptide sequence of 3 glycine residues (164-166) suggesting that it may comprise two distinct functional domains; thus peptide 167-184 was synthesized to represent the carboxyl domain of this peptide. The results suggested that domains on either side of the three glycine residues contribute to the antigenic activity of the 156-184 peptide. Peptide 167-184 was strongly antigenic, although less so than the 156-184 peptide. It should be noted, however, that the hexapeptide APGGGF (162-167) had significant antigenic activity in hexapeptide screening assays. Less antigenic activity was evident with a peptide representing residues 197-211, and only

limited activity was found with the 2-17 peptide. The degree of reactivity of each of the human sera with individual peptides was proportionate to the degree of binding of antibodies in pin-based hexapeptide ELISAs (Table 3). Serum AD099 did not react with any of the four abovementioned bulk-synthesized peptides, consistent with the fact that its reactivity with hexapeptides suggested dominant (but relatively weak) reactivity in the region 180-193. Figure 3 presents bar charts illustrating the reactivity of oligopeptides comprising amino acids 2-17, 58-78, 82-102, 123-143, 156-184 or 197-211 of HDAg with anti-HD positive and negative sera. Study of these charts reveals that many anti-HD positive sera react with more than one antigenic domain of HDAg. For example, positive serum 6 reacted strongly with oligopeptides 58-78, 82-102, 123-143 and 156-184.

Oligopeptide 2-17 was the least reactive of the six peptides treated in Figure 3. Additionally, oligopeptide 197-211 reacted strongly only with positive serum 2. Oligopeptide 156-184 wa the most reliable antigen, but several sera reacted much more strongly with 82-102 than with 156-184. Oligopeptide 123-143 showed a pattern of reactivity similar to that of 156-184, but with fewer strong reactions.

None of the six peptides evinced significant reactivity with the seventeen anti-HD negative sera.

The data presented in Table 3 and in Figure 3 indicate that synthetic peptides might be useful diagnostic reagents that could be employed in new assays for anti-HD antibodies. Studies with the 156-184 peptide suggest that it is recognized by IgG antibody present in approximately 90% of anti-HD positive sera, and that it may provide the basis for an antibody test that is significantly more sensitive than existing competitive immunoassays. Further studies of the immune response to this and other HDAg peptides will help in

determining whether there are differences in the fine structure (e.g., disease stage) of the B cell response to this protein in acute and chronic HDV infections, and in assessing the isotype specific responses to this antigen.

Although current understanding of the HDV-specific immunity is limited, previous infection of hepatitis B-positive chimpanzees with HDV has been shown to result in protection against severe hepatitis following rechallenge to HDV (23). Although it is possible that antibodies to HDAg may play a role in mediating such protection (as antibodies to hepatitis B core protein may offer some protection against hepatitis B infection), it is likely that T-cell-mediated immunity is also important (particularly cytotoxic T-cells). The antigenic peptides described herein may thus be capable of providing protection against HDV disease if administered as immunogenic conjugates with carrier proteins such as hepatitis B core protein. Because peptide 156-184 contains predicted amphipathic helical structures (20) at residues 150-159, 170-177 and 179-183, we determined its ability to induce antibody as a free peptide and as a peptide coupled to keyhole limpet hemocyanin (KLH) (Table 8). The peptide and peptide conjugate were administered to New Zealand white rabbits using Freund's adjuvant (complete and incomplete). The demonstrated immunogenicity of peptide 156-184 in the absence of coupling to a carrier protein suggests that this peptide contains important T-cell determinants as well as B-cell epitopes, and thus might be a particularly useful component in an HDV vaccine.

Table 1. Anti-HD Positive and Control Human Sera.

Serum	Age/Sex	HBsAg	Anti-HD*	Screening Dilution in Hexapeptide ELISA
AD062	28/M	+	1:62,500 (10^{-4})	1:6000
AF043	24/M	+	1:12,500 (10^{-3})	1:8000
AC036	31/M	+	1:62,500 (10^{-4})	1:8000
AC039	20/M	+	$\geq$1:312,500 (10^{-4})	1:8000
AD099	15/M	+	1:62,500 (10^{-3})	1:7000
A008	33/M	-	<1:100 ($<10^{-0}$)	1:8000
A073	-/M	+	<1:100 ($<10^{-0}$)	1:8000

* Highest dilution yielding 50% inhibition in competitive inhibition radioimmunoassay with woodchuck liver-derived HDAg (comparative endpoint titer in commercial ELISA).

Table 2. Antigenic Domains of HDAg Defined by Overlapping Hexapeptides with Antigenic Activity

	Antigenic Domains of HDAg							
Serum	1-7	63-74	86-92	94-100	121-128*	159-172	174-195	197-207
AD062	-	-	-	2	-	2	3	2
AF043	-	-	-	-	-	-	2	-
AC036	1	2	-	-	-	3	3	2
AC039	-	3	2	1	-	2	5	3
AD099	-	-	-	-	-	-	5	-
WC862	2	4	-	-	2	-	-	3
A008	-	-	-	-	-	-	-	-
A073	-	-	-	-	-	-	-	-

Note: Antigenic domains were defined by overlapping or contiguous hexapeptides with antigenic activity determined by screening against the panel of human sera (see Figure 3). Shown in the table are the number of hexapeptides within each domain that were determined to have antigenic activity with each individual serum specimen (absorbance > 0.2 for human sera, or > 0.5 for woodchuck serum).

* Recognized only by woodchuck serum.

Table 2A. Comparison of Antigenic Domains and Tested Peptides

Antigenic Domain	Substantially Homologous Peptides Tested
HD(1-7)	(Cys)-HD(2-17)
HD(63-74)	(Cys)-HD(58-78)
HD(86-92)	(Cys)-HD(82-102)
HD(94-100)	ditto
HD(121-128)	(Cys)-HD(123-143)
HD(159-172)	(Cys)-HD(156-184) HD(167-184)
HD(174-195)	none tested
HD(197-207)	HD(198-212)

Table 3. Detection of Anti-HD by Synthetic Peptide ELISA

Serum	Peptide Antigen 2-17	156-184	167-184	197-211
AD062	.07 ($<10^2$)*	>2.95 ($>10^6$)	>2.95 (10^5)	0.34 (10^2)
AF043	.06 ($<10^2$)	>2.95 (10^5)	1.29 (10^3)	0.28 (10^2)
AC036	.33 (10^2)	>2.95 ($>10^6$)	>2.95 ($>10^5$)	2.53 (10^2)
AC039	.09 ($<10^2$)	>2.95 ($>10^6$)	>2.95 (10^5)	>2.95 (10^4)
AD099	.09 ($<10^2$)	.04 ($<10^2$)	0.05 ($<10^2$)	0.03 ($<10^4$)
A008	.01 ($<10^2$)	.02 ($<10^2$)	.02 ($<10^2$)	.02 ($<10^2$)
A073	.01 ($<10^2$)	.05 ($<10^2$)	.03 ($<10^2$)	.02 ($<10^2$)

*OD490 at 1:100 serum dilution (endpoint titer)

Table 4: Predicted Antigenic Sites of HDAg

PEPTIDESTRUCTURE of: humanhdv.pep check: 6004 from: 1 to: 214

TRANSLATE of: human.frg /rev check: 1646 from: 1 to: 645

Hydrophilicity (Kyte-Doolittle) averaged over a window of: 7
Surface Probability according to Emini
Chain Flexibility according to Karplus-Schulz
Secondary Structure according to Chou-Fasman
Secondary Structure according to Garnier-Osguthorpe-Robson
Antigenicity Index according to Jameson-Wolf

Position numbers corresponding to preferred peptides of Figure 1 are boldfaced. Asterisks mark the antigenic domains of Table 2.

Pos	AA	HyPhil	SurfPr	FlexPr	CF-Pred	GORPred	AI-Ind ..
1*	M	1.050	2.986	1.000	.	.	0.900
2*	S	1.540	5.504	1.000	t	.	1.100
3*	R	2.033	14.087	1.000	t	.	1.100
4*	S	2.386	39.822	1.000	t	.	1.100
5*	E	3.214	97.659	1.081	.	T	1.300
6*	R	3.600	62.493	1.071	.	T	1.300
7*	R	3.600	140.907	1.063	.	T	1.300
8	K	3.543	50.619	1.061	t	T	1.500
9	D	3.100	13.096	1.067	t	T	1.500
10	R	3.100	13.096	1.076	T	T	1.700
11	G	2.957	8.671	1.090	T	.	1.300
12	G	2.900	8.671	1.093	t	.	1.100
13	R	1.757	2.088	1.078	H	.	0.900
14	E	0.571	2.352	1.058	H	.	0.900
15	D	1.014	6.547	1.031	H	.	0.900
16	I	1.457	4.749	1.003	H	H	0.900
17	L	0.943	2.416	0.992	H	H	0.750
18	E	-0.157	0.945	0.984	H	H	-0.300
19	Q	-0.543	1.741	0.980	H	H	-0.450
20	W	0.157	1.545	0.988	H	H	0.450
21	V	1.343	2.146	1.001	H	H	0.900
22	S	1.400	3.217	1.027	t	H	1.100
23	G	1.457	9.549	1.054	t	.	1.100
24	R	0.786	11.121	1.063	t	.	1.100
25	K	1.886	18.058	1.065	H	.	0.900
26	K	2.271	50.268	1.052	H	.	0.900
27	L	1.671	14.650	1.030	H	H	0.900
28	E	1.529	9.701	1.024	H	H	0.900
29	E	1.614	8.919	1.017	H	H	0.900
30	L	1.557	19.583	1.018	H	H	0.900
31	E	1.557	7.925	1.026	H	H	0.900
32	R	1.700	11.004	1.029	H	H	0.900
33	D	1.757	41.067	1.034	H	H	0.900
34	L	1.757	16.619	1.029	H	H	0.900
35	R	1.814	18.076	1.031	H	H	0.900

36	K	1.729	30.723	1.037	H	H	0.900
37	L	1.786	114.656	1.041	H	H	0.900
38	K	1.686	27.605	1.054	H	H	0.900
39	K	1.600	27.605	1.060	H	H	0.900
40	K	1.600	103.017	1.062	H	H	0.900
41	I	1.600	27.605	1.056	H	H	0.900
42	K	1.543	18.278	1.050	H	H	0.900
43	K	1.486	12.103	1.045	H	H	0.900
44	L	1.429	32.167	1.038	H	H	0.900
45	E	2.571	18.769	1.047	H	H	0.900
46	E	2.243	9.385	1.057	H	T	1.300
47	D	1.814	11.799	1.056	t	T	1.500
48	N	1.814	4.775	1.053	t	.	1.100
49	P	1.371	1.715	1.035	T	T	1.700
50	W	1.371	1.701	1.010	T	T	1.700
51	L	0.229	0.645	1.001	.	.	0.450
52	G	0.286	1.291	1.005	.	T	1.000
53	N	0.114	0.911	1.013	.	T	0.850
54	I	-0.657	0.753	1.018	.	B	-0.450
55	K	-0.757	0.701	1.012	.	B	-0.450
56	G	-0.757	0.286	0.995	.	B	-0.600
57	I	-0.700	1.290	0.982	.	B	-0.450
58	I	0.500	1.290	0.988	.	B	0.750
59	G	0.443	3.191	1.011	.	B	0.600
60	K	0.943	14.417	1.038	.	.	0.900
61	K	2.086	38.319	1.059	t	.	1.100
62	D	2.786	38.319	1.070	t	T	1.500
63*	K	3.229	25.372	1.077	T	T	1.700
64*	D	2.729	6.035	1.077	T	T	1.700
65*	G	1.914	2.769	1.081	t	T	1.500
66*	E	1.643	1.384	1.078	t	T	1.500
67*	G	1.314	1.176	1.072	.	.	0.900
68*	A	0.557	1.335	1.068	.	.	0.900
69*	P	1.057	2.016	1.066	.	H	0.900
70*	P	1.114	8.476	1.068	t	H	1.100
71*	A	0.514	8.415	1.063	t	H	1.100
72*	K	1.414	15.473	1.049	H	H	0.900
73*	K	0.914	10.065	1.024	H	H	0.900
74*	L	1.186	21.940	1.000	H	H	0.900
75	R	1.943	14.634	0.987	H	H	0.750
76	M	1.114	4.760	0.986	H	H	0.750
77	D	1.057	11.761	0.992	H	H	0.750
78	Q	0.957	2.832	0.993	H	H	0.750
79	M	0.814	5.122	0.983	H	H	0.750
80	E	0.829	2.350	0.977	H	H	0.750
81	I	0.386	0.838	0.978	H	H	0.300
82	D	0.114	1.288	0.988	H	H	0.450
83	A	1.029	1.789	1.011	H	.	0.900
84	G	1.086	8.081	1.031	.	.	0.900
85	P	2.371	12.628	1.044	T	.	1.300
86*	R	2.100	23.394	1.055	T	.	1.300
87*	K	1.814	26.354	1.056	.	.	0.900
88*	R	2.400	48.461	1.049	.	.	0.900
89*	P	2.229	12.537	1.049	.	.	0.900
90*	L	1.643	2.982	1.044	.	T	1.300

91*	R	0.686	0.803	1.041	T	T	1.550
92*	G	0.143	0.702	1.044	T	T	1.250
93	G	0.414	1.541	1.034	.	T	1.000
94*	F	1.514	1.676	1.029	h	.	0.900
95*	T	1.371	4.664	1.037	h	.	0.900
96*	D	1.957	18.030	1.043	h	.	0.900
97*	K	2.400	48.574	1.064	h	.	0.900
98*	E	3.300	65.413	1.082	h	H	0.900
99*	R	3.657	54.727	1.082	h	H	0.900
100*	Q	3.800	50.317	1.082	h	H	0.900
101	D	3.886	69.868	1.066	h	H	0.900
102	H	4.029	69.868	1.048	h	H	0.900
103	R	3.943	104.751	1.042	h	H	0.900
104	R	3.186	48.054	1.032	h	H	0.900
105	R	2.143	26.160	1.025	h	H	0.900
106	K	2.186	18.839	1.021	h	H	0.900
107	A	2.043	11.956	1.014	h	H	0.900
108	L	1.957	13.004	1.020	h	H	0.900
109	E	1.957	11.956	1.036	h	H	0.900
110	N	1.957	44.298	1.056	.	H	1.300
111	K	2.714	110.263	1.068	T	H	1.300
112	R	2.714	44.623	1.063	T	H	0.900
113	K	2.329	31.184	1.053	.	H	0.900
114	Q	1.943	12.716	1.045	.	.	0.900
115	L	1.443	3.290	1.050	T	.	1.150
116	S	0.857	0.783	1.073	T	T	1.700
117	S	0.857	1.173	1.099	T	T	1.400
118	G	0.471	1.785	1.112	T	.	1.000
119	G	0.471	1.173	1.108	t	.	0.800
120	K	0.471	1.173	1.086	t	.	1.100
121*	S	1.000	4.535	1.058	H	.	0.900
122*	L	1.443	12.623	1.041	H	.	0.900
123*	S	1.886	8.358	1.042	H	H	0.900
124*	R	1.829	13.572	1.053	H	H	0.900
125*	E	2.214	33.537	1.070	H	H	0.900
126*	E	3.257	54.458	1.081	H	H	0.900
127*	E	2.600	15.872	1.075	H	H	0.900
128*	E	2.514	23.970	1.067	H	H	0.900
129	E	2.657	33.284	1.050	H	H	0.900
130	L	1.614	13.470	1.033	H	H	0.900
131	K	1.214	8.888	1.029	H	H	0.900
132	R	1.214	8.888	1.027	H	H	0.900
133	L	1.214	21.962	1.032	H	H	0.900
134	T	2.257	12.921	1.047	H	H	0.900
135	E	2.200	9.305	1.058	H	H	0.900
136	E	2.114	34.727	1.069	H	H	0.900
137	D	3.300	73.081	1.072	H	H	0.900
138	E	3.700	73.081	1.075	H	H	0.900
139	K	3.843	101.477	1.072	H	H	0.900
140	R	3.986	158.579	1.065	H	H	0.900
141	E	2.843	53.015	1.052	H	H	0.900
142	R	2.086	14.309	1.026	H	H	0.900
143	R	1.586	3.702	1.004	H	.	0.750
144	I	1.171	2.795	0.987	H	.	0.750
145	A	0.786	1.240	0.990			

146	G	-0.457	0.310	1.004	.	.	-0.450
147	P	-1.043	0.333	1.018	T	.	-0.050
148	S	-0.343	0.294	1.029	T	.	0.250
149	V	-0.686	0.284	1.026	T	.	-0.050
150	G	-0.243	0.332	1.023	T	.	0.250
151	G	-0.243	0.407	1.022	.	.	-0.150
152	V	-0.900	0.474	1.017	.	.	-0.450
153	N	0.200	1.318	1.020	.	.	0.600
154	P	0.200	1.318	1.030	t	.	0.800
155	L	0.200	1.363	1.041	t	.	0.800
156	E	0.914	0.953	1.069	t	T	1.350
157	G	1.057	1.752	1.094	t	T	1.500
158	G	0.886	1.555	1.104	t	T	1.500
159*	S	1.171	0.634	1.104	T	.	1.150
160*	R	0.900	1.332	1.086	T	.	1.300
161*	G	0.900	1.332	1.075	t	.	1.100
162*	A	0.900	0.777	1.076	.	.	0.750
163*	P	0.843	0.201	1.084	T	.	1.150
164*	G	-0.200	0.209	1.086	T	T	0.650
165*	G	-0.857	0.178	1.068	T	T	0.350
166*	G	-0.371	0.178	1.040	t	.	0.050
167*	F	-0.486	0.306	1.012	.	B	-0.450
168*	V	-0.814	0.418	0.998	.	B	-0.600
169*	P	-0.371	1.173	1.003	t	B	0.200
170*	S	-0.371	1.127	1.010	t	B	0.200
171*	M	-0.571	1.127	1.011	.	B	-0.300
172*	Q	0.257	1.127	1.019	.	.	0.600
173	G	0.529	1.829	1.025	.	.	0.900
174*	V	0.529	2.294	1.042	.	.	0.900
175*	P	1.029	1.719	1.069	.	.	0.900
176*	E	0.129	1.789	1.077	h	.	0.600
177*	S	-0.186	2.099	1.071	h	.	0.000
178*	P	1.057	3.860	1.049	h	.	0.900
179*	F	0.929	2.547	1.019	h	.	0.900
180*	A	0.486	1.486	1.014	h	.	0.600
181*	R	0.871	1.967	1.029	h	.	0.900
182*	T	0.700	1.890	1.052	t	.	1.100
183*	G	0.557	1.877	1.070	t	.	1.100
184*	E	1.314	1.201	1.061	h	.	0.900
185*	G	0.029	0.608	1.036	h	.	0.450
186*	L	0.571	2.352	1.010	h	.	0.900
187*	D	0.571	0.845	1.001	h	.	0.750
188*	I	0.186	1.448	1.020	h	T	1.000
189*	R	0.629	3.605	1.055	h	T	1.300
190*	G	1.229	1.458	1.092	.	T	1.300
191*	S	0.329	1.630	1.104	t	T	1.200
192*	Q	1.200	0.886	1.087	t	.	0.950
193*	G	0.686	1.256	1.045	t	.	1.100
194*	F	1.129	1.812	0.995	.	.	0.750
195*	P	0.371	0.601	0.956	t	T	0.900
196	W	-0.671	0.677	0.931	t	T	0.000
197*	D	-1.129	0.677	0.920	.	B	-0.600
198*	I	-0.500	0.677	0.917	b	B	-0.600
199*	L	-0.986	0.543	0.924	b	B	-0.600
200*	F	-0.614	0.543	0.943	b	B	-0.600

201*	P	-0.886	1.226	0.972	b	T	-0.050
202*	A	-0.014	2.287	1.010	b	T	0.400
203*	D	0.129	2.287	1.033	.	.	0.600
204*	P	0.643	1.865	1.041	t	.	1.100
205*	P	0.643	3.455	1.042	T	T	1.700
206*	F	1.400	3.917	1.037	T	T	1.700
207*	S	1.014	3.194	1.047	.	.	0.900
208	P	0.429	0.961	1.054	t	T	1.050
209	Q	0.843	3.571	1.059	T	T	1.700
210	S	1.471	4.378	1.052	T	T	1.700
211	C	1.857	5.840	1.000	t	T	1.500
212	R	1.900	3.145	1.000	.	.	0.900
213	P	1.580	2.771	1.000	.	T	1.300
214	Q	1.775	6.615	1.000	.	T	1.300

Table 5: HDAg Hexapeptide Screening Results

		Serum				
		AC039 1:8000	AC036 1:8000	AF043 1:8000	AD099 1:7000	AD062 1:6000
Residue						
1	M	0.140	0.122			
2	S	0.170	0.291	0.111	0.118	0.113
3	R	0.137	0.103			
4	S	0.132	0.094			
5	E	0.132	0.101			
6	R	0.156	0.102			
7	R	0.153	0.095			
8	K	0.141	0.093			
9	D	0.127	0.093			
10	R	0.126	0.097			
11	G	0.142	0.090			
12	G	0.138	0.100			
13	R	0.135	0.095			
14	E	0.145	0.099			
15	D	0.160	0.103			
16	I	0.166	0.106			
17	L	0.150	0.109			
18	E	0.134	0.097			
19	Q	0.160	0.100			
20	W	0.144	0.094			
21	V	0.134	0.090	0.099	0.106	0.104
22	S	0.139	0.096	0.117	0.116	0.120
23	G	0.150	0.092	0.129	0.110	0.112
24	R	0.142	0.097	0.106	0.105	0.120
25	K	0.150	0.101	0.112	0.115	0.137
26	K	0.132	0.098	0.109	0.110	0.119
27	L	0.141	0.097	0.114	0.116	0.121
28	E	0.140	0.096	0.111	0.110	0.118
29	E	0.155	0.113	0.123	0.142	0.151
30	L	0.137	0.103	0.125	0.141	0.150
31	E	0.133	0.095	0.113	0.121	0.119
32	R	0.141	0.098	0.122	0.132	0.128
33	D	0.130	0.093	0.106	0.116	0.108
34	L	0.118	0.091	0.102	0.115	0.112
35	R	0.126	0.088	0.113	0.114	0.127
36	K	0.135	0.097	0.110	0.113	0.114
37	L	0.119	0.091	0.103	0.113	0.107
38	K	0.104	0.092	0.098	0.108	0.100
39	K	0.112	0.091	0.106	0.122	0.109
40	K	0.126	0.099	0.109	0.118	0.115
41	I	0.124	0.097	0.103	0.103	0.116
42	K	0.127	0.101	0.105	0.107	0.121
43	K	0.146	0.092	0.107	0.111	0.135
44	L	0.129	0.089	0.105	0.110	0.120
45	E	0.137	0.097	0.111	0.106	0.115
46	E	0.145	0.099	0.119	0.133	0.126
47	D	0.134	0.093	0.125	0.168	0.120

48	N	0.151	0.102	0.119	0.134	0.125
49	P	0.152	0.098	0.121	0.133	0.129
50	W	0.122	0.092	0.101	0.098	0.100
51	L	0.132	0.098	0.100	0.103	0.101
52	G	0.137	0.100	0.108	0.113	0.111
53	N	0.138	0.107	0.117	0.136	0.133
54	I	0.144	0.105	0.121	0.126	0.135
55	K	0.121	0.094	0.103	0.104	0.107
56	G	0.108	0.081	0.094	0.100	0.099
57	I	0.129	0.098	0.103	0.105	0.105
58	I	0.119	0.095	0.106	0.111	0.124
59	G	0.135	0.095	0.122	0.125	0.130
60	K	0.157	0.105	0.124	0.122	0.125
61	K	0.153	0.098	0.108	0.107	0.113
62	D	0.159	0.106	0.114	0.122	0.123
63	K	0.153	0.262	0.114	0.120	0.118
64	D	0.148	0.525	0.111	0.111	0.121
65	G	0.140	0.177	0.115	0.115	0.131
66	E	0.148	0.184	0.118	0.123	0.136
67	G	0.201	0.099	0.100	0.106	0.102
68	A	0.248	0.101	0.109	0.110	0.109
69	P	0.269	0.102	0.113	0.111	0.108
70	P	0.126	0.095	0.111	0.114	0.117
71	A	0.147	0.098	0.131	0.120	0.119
72	K	0.139	0.096	0.115	0.110	0.111
73	K	0.146	0.099	0.117	0.120	0.124
74	L	0.164	0.107	0.116	0.120	0.124
75	R	0.153	0.101	0.108	0.118	0.124
76	M	0.155	0.100	0.112	0.123	0.135
77	D	0.163	0.107	0.121	0.131	0.129
78	Q	0.138	0.105	0.108	0.114	0.114
79	M	0.121	0.100	0.102	0.112	0.112
80	E	0.112	0.085	0.102	0.126	0.105
81	I	0.133	0.095	0.109	0.108	0.122
82	D	0.156	0.106	0.115	0.121	0.136
83	A	0.141	0.087	0.119	0.109	0.114
84	G	0.138	0.117	0.123	0.118	0.122
85	P	0.125	0.168	0.122	0.118	0.117
86	R	0.267	0.135	0.125	0.133	0.133
87	K	0.200	0.105	0.119	0.132	0.128
88	R	0.124	0.099	0.112	0.124	0.116
89	P	0.132	0.109	0.128	0.146	0.159
90	L	0.137	0.111	0.126	0.149	0.163
91	R	0.127	0.095	0.110	0.114	0.134
92	G	0.125	0.089	0.106	0.106	0.112
93	G	0.133	0.099	0.103	0.108	0.126
94	F	0.479	0.105	0.103	0.109	0.215
95	T	0.139	0.101	0.116	0.127	0.207
96	D	0.146	0.108	0.122	0.131	0.143
97	K	0.130	0.099	0.109	0.120	0.120
98	E	0.124	0.094	0.113	0.124	0.121
99	R	0.120	0.101	0.121	0.132	0.128
100	Q	0.127	0.100	0.122	0.130	0.130
101	D	0.123	0.099	0.123	0.126	0.120
102	H	0.132	0.109	0.123	0.133	0.130

103	R	0.130	0.108	0.128	0.139	0.137
104	R	0.129	0.106	0.122	0.132	0.139
105	R	0.136	0.090	0.115	0.128	0.134
106	K	0.118	0.101	0.111	0.121	0.125
107	A	0.138	0.104	0.125	0.140	0.130
108	L	0.119	0.097	0.110	0.112	0.106
109	E	0.158	0.121	0.135	0.163	0.142
110	N	0.163	0.107	0.142	0.151	0.146
111	K	0.141	0.112	0.132	0.143	0.132
112	R	0.141	0.107	0.126	0.142	0.129
113	K	0.129	0.110	0.127	0.146	0.139
114	Q	0.126	0.093	0.110	0.120	0.126
115	L	0.122	0.102	0.108	0.114	0.120
116	S	0.128	0.101	0.111	0.126	0.130
117	S	0.139	0.095	0.124	0.127	0.131
118	G	0.132	0.111	0.124	0.120	0.125
119	G	0.128	0.104	0.131	0.119	0.134
120	K	0.154	0.114	0.136	0.146	0.146
121	S	0.121	0.097	0.114	0.112	0.122
122	L	0.121	0.096	0.114	0.113	0.123
123	S	0.116	0.096	0.116	0.117	0.126
124	R	0.116	0.095	0.117	0.114	0.123
125	E	0.142	0.102	0.118	0.121	0.145
126	E	0.136	0.098	0.118	0.118	0.135
127	E	0.138	0.098	0.113	0.116	0.119
128	E	0.137	0.100	0.126	0.127	0.131
129	E	0.138	0.094	0.131	0.162	0.140
130	L	0.120	0.096	0.115	0.141	0.139
131	K	0.128	0.101	0.114	0.137	0.153
132	R	0.116	0.096	0.126	0.113	0.137
133	L	0.137	0.096	0.116	0.117	0.126
134	T	0.129	0.100	0.118	0.119	0.139
135	E	0.139	0.101	0.114	0.115	0.131
136	E	0.129	0.100	0.114	0.116	0.129
137	D	0.126	0.093	0.112	0.116	0.119
138	E	0.122	0.092	0.117	0.121	0.120
139	K	0.128	0.100	0.124	0.135	0.131
140	R	0.130	0.099	0.125	0.135	0.143
141	E	0.130	0.094	0.118	0.137	0.138
142	R	0.128	0.112	0.122	0.142	0.137
143	R	0.119	0.093	0.113	0.119	0.123
144	I	0.131	0.103	0.112	0.121	0.122
145	A	0.131	0.102	0.116	0.135	0.131
146	G	0.113	0.093	0.109	0.121	0.118
147	P	0.133	0.111	0.115	0.126	0.128
148	S	0.136	0.104	0.120	0.141	0.136
149	V	0.112	0.092	0.109	0.107	0.115
150	G	0.141	0.103	0.113	0.116	0.134
151	G	0.121	0.127	0.108	0.115	0.126
152	V	0.128	0.159	0.112	0.131	0.125
153	N	0.133	0.099	0.117	0.197	0.129
154	P	0.120	0.092	0.111	0.199	0.111
155	L	0.126	0.096	0.120	0.120	0.127
156	E	0.111	0.093	0.110	0.110	0.112
157	G	0.119	0.096	0.111	0.117	0.117

158	G	0.124	0.146	0.114	0.115	0.119
159	S	0.146	0.360	0.107	0.111	0.113
160	R	0.147	0.348	0.109	0.108	0.112
161	G	0.131	0.102	0.108	0.116	0.119
162	A	0.516	0.112	0.137	0.138	0.146
163	P	0.118	0.102	0.117	0.115	0.113
164	G	0.132	0.121	0.125	0.126	0.133
165	G	0.137	0.102	0.122	0.122	0.131
166	G	0.140	0.115	0.117	0.152	0.407
167	F	0.471	1.736	0.137	0.165	0.369
168	V	0.154	0.117	0.139	0.151	0.139
169	P	0.125	0.098	0.112	0.183	0.121
170	S	0.120	0.098	0.110	0.117	0.120
171	M	0.139	0.100	0.116	0.163	0.125
172	Q	0.128	0.097	0.111	0.114	0.117
173	G	0.125	0.095	0.109	0.143	0.119
174	V	0.217	0.309	1.295	0.110	0.419
175	P	0.776	0.929	1.005	0.109	0.858
176	E	0.574	0.393	0.117	0.241	0.180
177	S	0.130	0.092	0.118	0.139	0.642
178	P	0.119	0.094	0.115	0.120	0.122
179	F	0.116	0.096	0.115	0.089	0.124
180	A	0.124	0.101	0.118	0.344	0.130
181	R	0.125	0.100	0.117	0.225	0.126
182	T	0.129	0.097	0.115	0.124	0.130
183	G	0.136	0.101	0.120	0.145	0.132
184	E	0.138	0.103	0.124	0.398	0.142
185	G	0.124	0.100	0.115	0.141	0.124
186	L	0.124	0.092	0.116	0.192	0.122
187	D	0.117	0.101	0.111	0.136	0.121
188	I	0.123	0.100	0.114	0.317	0.119
189	R	0.311	0.094	0.111	0.115	0.110
190	G	0.217	0.104	0.115	0.128	0.121
191	S	0.160	0.099	0.118	0.123	0.120
192	Q	0.145	0.097	0.119	0.126	0.123
193	G	0.151	0.097	0.118	0.161	0.121
194	F	0.144	0.099	0.128	0.180	0.132
195	P	0.149	0.092	0.125	0.139	0.130
196	W	0.132	0.095	0.120	0.128	0.134
197	D	0.228	0.095	0.112	0.113	0.119
198	I	0.121	0.096	0.112	0.120	0.119
199	L	0.177	0.088	0.108	0.113	0.126
200	F	0.130	0.097	0.100	0.115	0.108
201	P	2.272	0.458	0.116	0.147	0.213
202	A	2.233	0.432	0.107	0.108	0.231
203	D	0.187	0.163	0.108	0.108	0.123
204	P	0.182	0.095	0.110	0.125	0.161
205	P	0.147	0.094	0.110	0.117	0.160
206	F	0.128	0.087	0.106	0.106	0.111
207	S	0.141	0.093	0.117	0.121	0.123
208	P	0.131	0.091	0.110	0.115	0.113
209	Q	0.128	0.092	0.111	0.123	0.117

Table 6. Examples of Two Antigenic Domains of HDAg Determined by Pin-Based Hexapeptide ELISA

A.

S R G A P G G G F V P S M Q	OD_{490}	Reactive Sera
S R G A P G	0.360	AC036
R G A P G G	0.348	AC036
A P G G G F	0.516	AC039
G F V P S M	0.407	AD062
F V P S M Q	0.859	AC036, AC039, AD062

B.

V P E S P F A R T G E G L D I R G S Q G F P	OD_{490}	Reactive Sera
V P E S P F	0.560	AC036,AC039, AF043,AD062
P E S P F A	0.892	AC036,AC039, AF043,AD062
E S P F A R	0.403	AC036,AC039, AD099
S P F A R T	0.642	AD062
A R T G E G	0.344	AD099
R T G E G L	0.225	AD099
E G L D I R	0.398	AD099
I R G S Q G	0.317	AD099
R G S Q G F	0.311	AC039
G S Q G F P	0.217	AC039

Antigenic domains were defined by overlapping or contiguous peptide sequence found to be reactive as hexapeptides when tested against any anti-HD positive serum. Only reactive hexapeptides are shown. Where the hexapeptide was reactive with more than one of the tested sera, the OD_{490} represents the mean absorbance: A) antigenic domain at residues 159-172, B) antigenic domain at residues 174-195. See also Table 2.

Table 7. Comparison of Predicted and Observed Antigenic Residues of HDAg

Method	Sens	Spec	Positive Predictive Value	Negative Predicted Value	kappa
Hydrophilicity	0.38	0.43	0.31	0.50	-.19
Surface Probability	0.33	0.45	0.30	0.49	-.21
Flexibility	0.65	0.56	0.51	0.70	.20
Antigenic Index	0.19	0.83	0.44	0.59	.02
Predicted Turn	0.45	0.67	0.49	0.64	.12

Sens = Sensitivity; Spec = Specificity

Table 8. Immunogenicity of HDV Peptides and Peptide Conjugates

Rabbit	Immunogen	Anti-peptide (cognate peptide)	Western Blot
LP-1	HD2-17/KLH	>=1:156,000	neg
LP-2	HD156-184/KLH	>=1:156,000	+
LP-3	HD197-211/KLH	>=1:156,000	neg
LP-4	HD156-184	>=1:1250	+
LP-5	HAV-VP4	n.d	neg

Immunogens were given to rabbits in Freund's complete adjuants, followed by subsequent booster immunizations in Freund's incomplete adjuvant. Rabbit LP-5 received a control peptide representing the putative UP4 capsid protein of hepatitis A virus. Sera collected from immunized rabbits were tested in ELISA assays against the cognate peptide (uncoupled) and by Western blot (1:100 serum dilution) against HDAg purified from an infected woodchuck.

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CLAIMS

1. A first peptide of ten to fifty amino acids having the sequence (Cys-)Ser-Arg-Ser-Glu-Arg-Arg-Lys-Asp-Arg-Gly-Gly-Arg-Glu-Asp-Ile-Leu or a peptide of ten to fifty amino acids which substantially inhibits the binding of said first peptide by human anti-hepatitis delta virus antibodies.

2. A first peptide of ten to fifty amino acids having the sequence (Cys-)Ile-Gly-Lys-Lys-Asp-Lys-Asp-Gly-Glu-Gly-Ala-Pro-Pro-Ala-Lys-Lys-Leu-Arg-Met-Asp-Gln or a peptide of ten to fifty amino acids which substantially inhibits the binding of said first peptide by human anti-hepatitis delta virus antibodies.

3. A first peptide of ten to fifty amino acids having the sequence (Cys-)Asp-Ala-Gly-Pro-Arg-Lys-Arg-Pro-Leu-Arg-Gly-Gly-Phe-Thr-Asp-Lys-Glu-Arg-Gln-Asp-His or a peptide of ten to fifty amino acids which substantially inhibits the binding of said first peptide by human anti-hepatitis delta virus antibodies.

4. A first peptide of ten to fifty amino acids having the sequence (Cys-)Ser-Arg-Glu-Glu-Glu-Glu-Glu-Leu-Lys-Arg-Leu-Thr-Glu-Glu-Asp-Glu-Lys-Arg-Glu-Arg-Arg or a peptide of ten to fifty amino acids which substantially inhibits the binding of said first peptide by human anti-hepatitis delta virus antibodies.

5. A first peptide of ten to fifty amino acids having the sequence (Cys-)Glu-Gly-Gly-Ser-Arg-Gly-Ala-Pro-Gly-Gly-Gly-Phe-Val-Pro-Ser-Met-Gln-Gly-Val-Pro-Glu-Ser-Pro-Phe-Ala-Arg-Thr-Gly-Glu or a peptide of ten to fifty amino acids which substantially inhibits the binding of said first peptide by human anti-hepatitis delta virus antibodies.

6. A first peptide of ten to fifty amino acids having the sequence
Phe-Val-Pro-Ser-Met-Gln-Gly-Val-Pro-Glu-Ser-Pro-Phe-Ala-Arg-Thr-Gly-Glu or a peptide of ten to fifty amino acids which substantially inhibits the binding of said first peptide by human anti-hepatitis delta virus antibodies.

7. A first peptide of ten to fifty amino acids having the sequence
Asp-Ile-Leu-Phe-Pro-Ala-Asp-Pro-Pro-Phe-Ser-Pro-Gln-Ser-Cysor a peptide of ten to fifty amino acids which substantially inhibits the binding of said first peptide by human anti-hepatitis delta virus antibodies.

8. A peptide of ten to fifty amino acids comprising one of the following sequences,

(a) (Cys-)Ser-Arg-Ser-Glu-Arg-Arg-Lys-Asp-Arg-Gly-Gly- Arg-Glu-Asp-Ile-Leu,

(b) (Cys-)Ile-Gly-Lys-Lys-Asp-Lys-Asp-Gly-Glu-Gly-Ala- Pro-Pro-Ala-Lys-Lys-Leu-Arg-Met-Asp-Gln,

(c) (Cys-)Asp-Ala-Gly-Pro-Arg-Lys-Arg-Pro-Leu-Arg-Gly- Gly-Phe-Thr-Asp-Lys-Glu-Arg-Gln-Asp-His,

(d) (Cys-)Ser-Arg-Glu-Glu-Glu-Glu-Glu-Leu-Lys-Arg-Leu- Thr-Glu-Glu-Asp-Glu-Lys-Arg-Glu-Arg-Arg, (e) (Cys-)Glu-Gly-Gly-Ser-Arg-Gly-Ala-Pro-Gly-Gly-Phe- Val-Pro-Ser-Met-Gln-Gly-Val-Pro-Glu-Ser-Pro-Phe-Ala-Arg-Thr-Gly-Glu,

(f) Phe-Val-Pro-Ser-Met-Gln-Gly-Val-Pro-Glu-Ser-Pro-Phe-Ala-Arg-Thr-Gly-Glu, or

(g) Asp-Ile-Leu-Phe-Pro-Ala-Asp-Pro-Pro-Phe-Ser-Pro-Gln-Ser-Cys,

or a peptide of ten to fifty amino acids which substantially inhibits the binding of one and only one of said peptides (a)-(g) by human anti-hepatitis delta virus antibodies.

9. In an immunoassay to detect the presence or measure the level of anti-hepatitis delta virus antibodies in a sample, the improvement which comprises use of one or more peptides according to claim 8 as an antigenic diagnostic reagent.

10. A vaccine comprising a peptide of claim 8 conjugated with an immunogenic carrier.

11. A peptide comprising a sequence substantially homologous with one and only one of the following antigenic domains of hepatitis delta antigen: 1-7, 63-74, 86-92, 94-100, 121-128, 159-172, 174-195 and 197-207.

12. A peptide comprising at least one of the 159-172, 174-195 and 197-207 antigenic domains of HDAg and consisting of no more than about 100 amino acids.

13. The method of claim 9 in which a plurality of such peptides are used as a diagnostic panel.

14. The vaccine of claim 10, wherein the peptide comprises a sequence substantially homologous with the hepatitis delta antigen sequence 156-184.

SUBSTITUTE SHEET

FIG 1.

Delta ORF-5 Peptides

#2218 D5/2-17 [N-terminus]

Cys-Ser-Arg-Ber-Glu-Arg-Lys-Asp-Arg-Gly-Arg-Glu-Asp-Ile-Leu
2 12

#2649 D5/58-78

Cys-Ile-Gly-Lys-Lys-Asp-Lys-Asp-Gly-Glu-Ala-Pro-Pro-Ala-Lys-Lys-Leu-Arg-Met-Asp-Gln
58 68 78

#2596 D5/82-102

Cys-Asp-Ala-Gly-Pro-Arg-Pro-Leu-Arg-Gly-Gly-Phe-Thr-Asp-Lys-Glu-Arg-Gln-Asp-His
82 92 102

#2602 D3/123-143

Cys-Ser-Arg-Glu-Glu-Glu-Glu-Leu-Lys-Arg-Leu-Thr-Glu-Glu-Asp-Glu-Lys-Arg-Glu-Arg-Arg
123 133 143

#2387 D5/156-184

Cys-Glu-Gly-Ser-Arg-Gly-Ala-Pro-Gly-Gly-Gly-Phe-Val-Pro-Ser-Met-Gln-Gly-Val-Pro-Glu
156 166 176
-Ser-Pro-Phe-Ala-Arg-Thr-Gly-Glu
184

#2391 D5/167-184

Phe-Val-Pro-Ser-Met-Gln-Gly-Val-Pro-Glu-Ser-Pro-Phe-Ala-Arg-Thr-Gly-Glu
167 176 184

#2269 D5/198-212 [C-terminus]

Asp-Ile-Leu-Phe-Pro-Ala-Asp-Pro-Pro-Phe-Ser-Pro-Gln-Ser-Cys
197 207

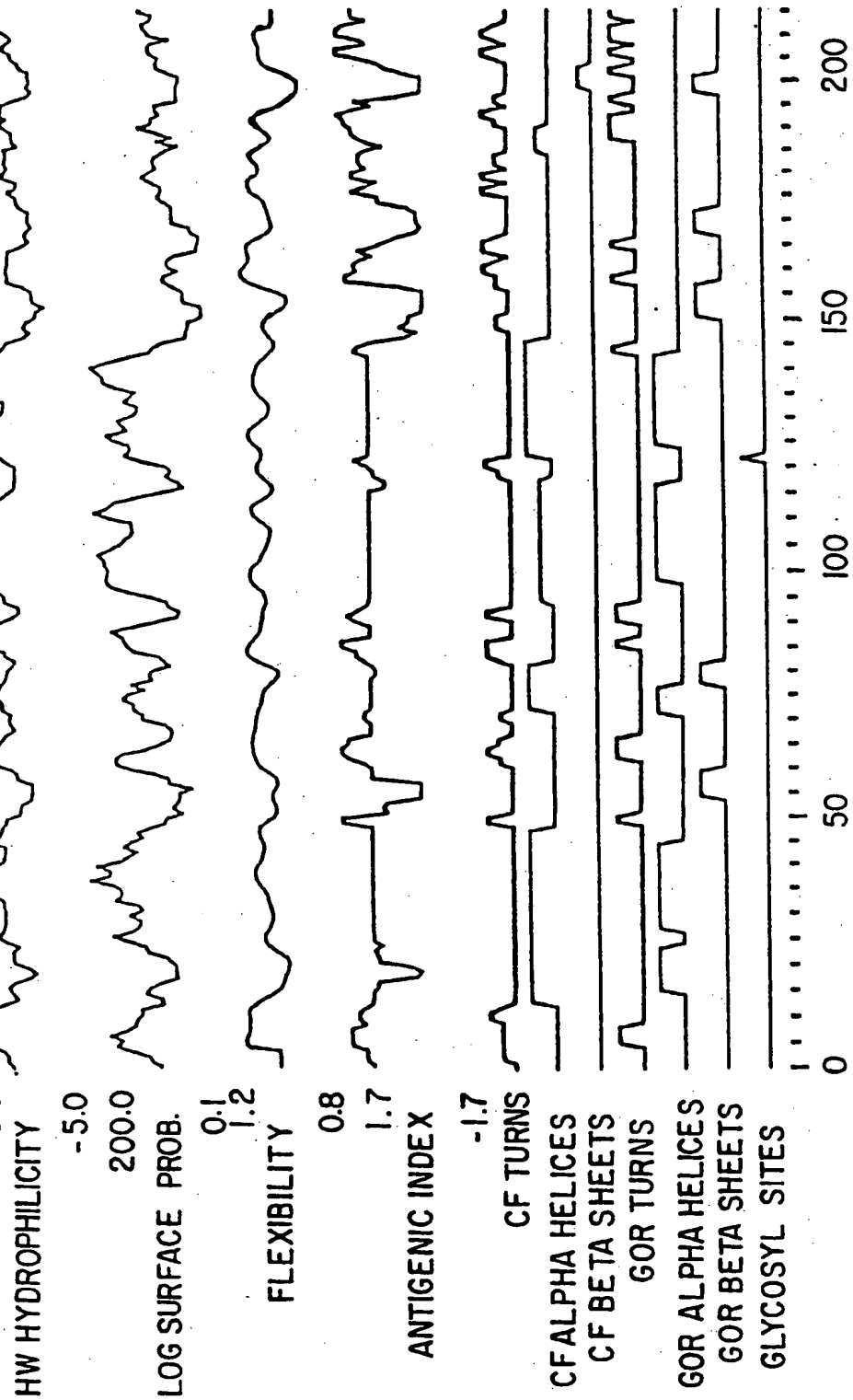
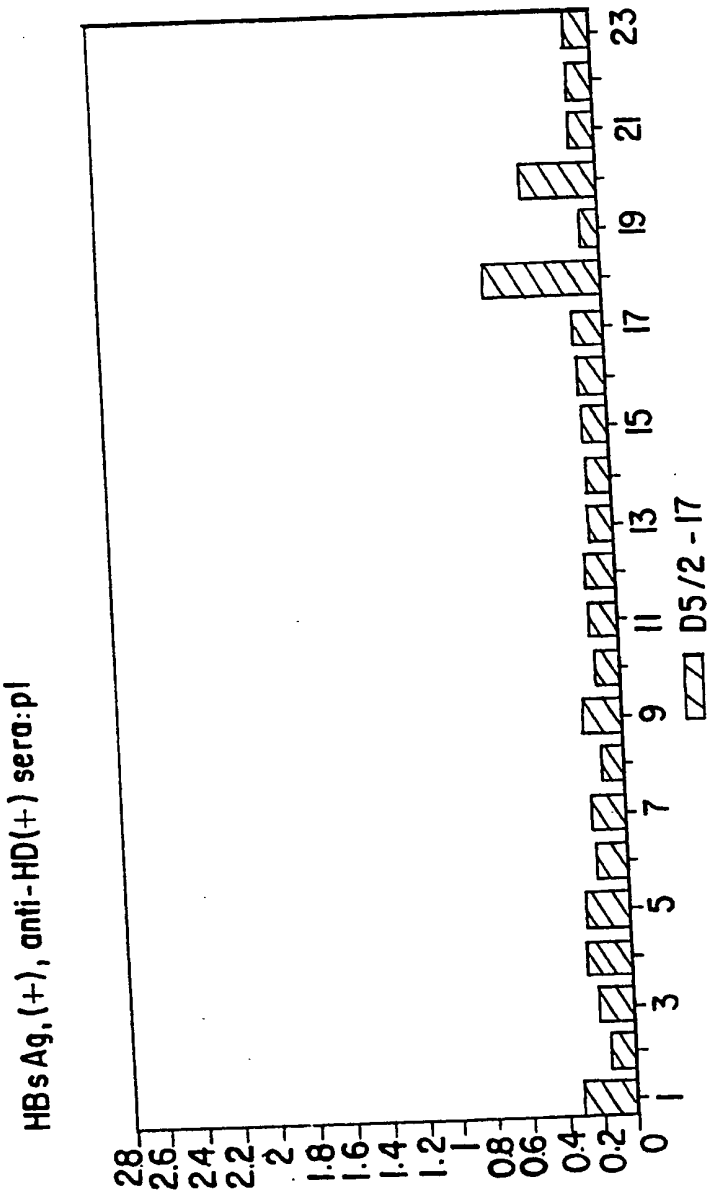

0
50
100
150
200
5.0
HW HYDROPHILICITY
-5.0
200.0
LOG SURFACE PROB.
0.1
1.2
FLEXIBILITY
0.8
1.7
ANTIGENIC INDEX
-1.7
CF TURNS
CF ALPHA HELICES
CF BETA SHEETS
GOR TURNS
GOR ALPHA HELICES
GOR BETA SHEETS
GLYCOSYL SITES
0
50
100
150
200

FIG. 2.


HBsAg,(+), anti-HD(+) sera:pl
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
2.8
1
3
5
7
9
11
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19
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23
D5/2-17

FIG. 3A.

FIG. 3B.

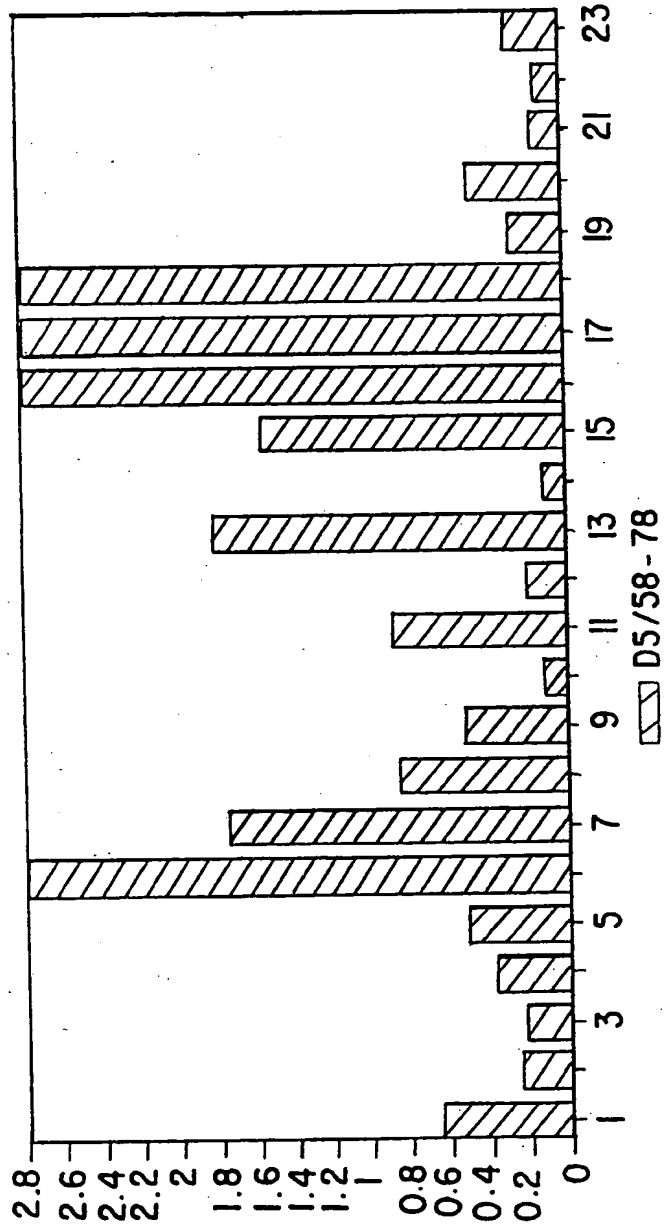
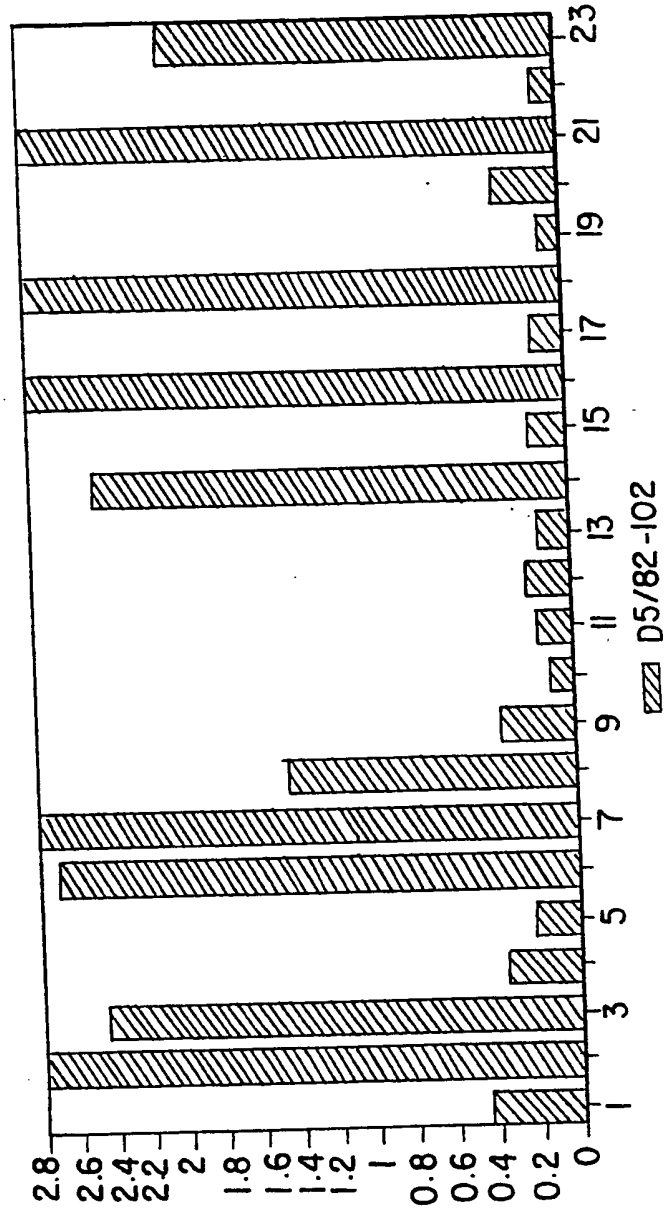

2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
3
5
7
9
11
13
15
17
19
21
23
D5/58-78

FIG. 3C.


2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
3
5
7
9
11
13
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17
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21
23
D5/82-102

FIG. 3D.

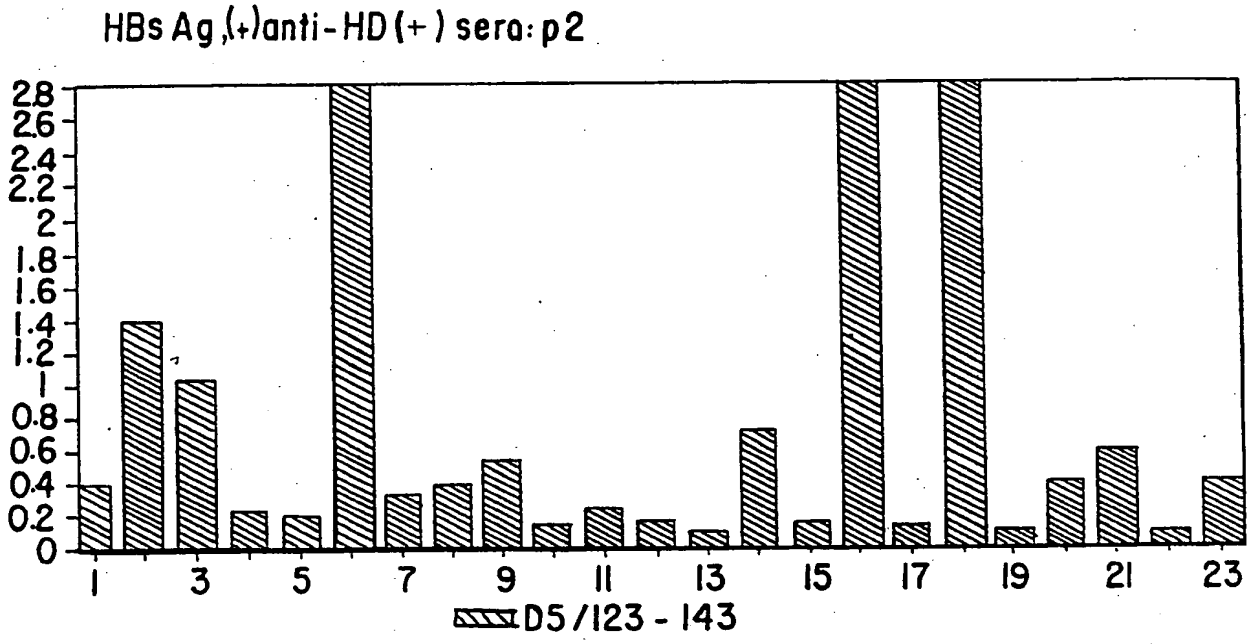

HBs Ag (+) anti-HD (+) sera: p2
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 3 5 7 9 11 13 15 17 19 21 23
D5/123 - 143

FIG. 3E.

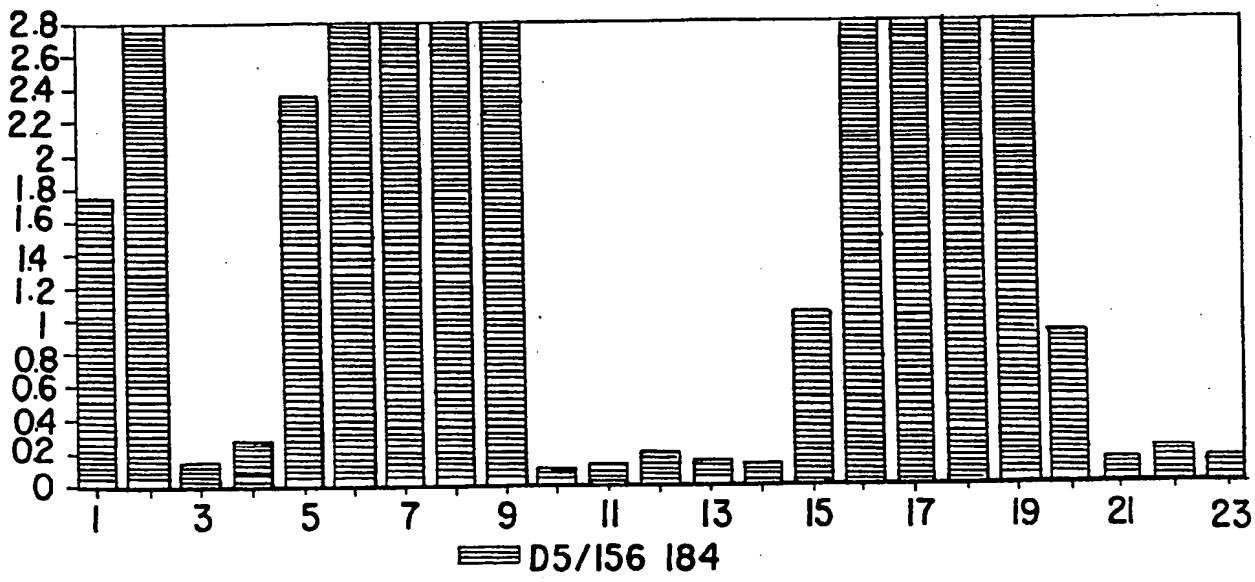
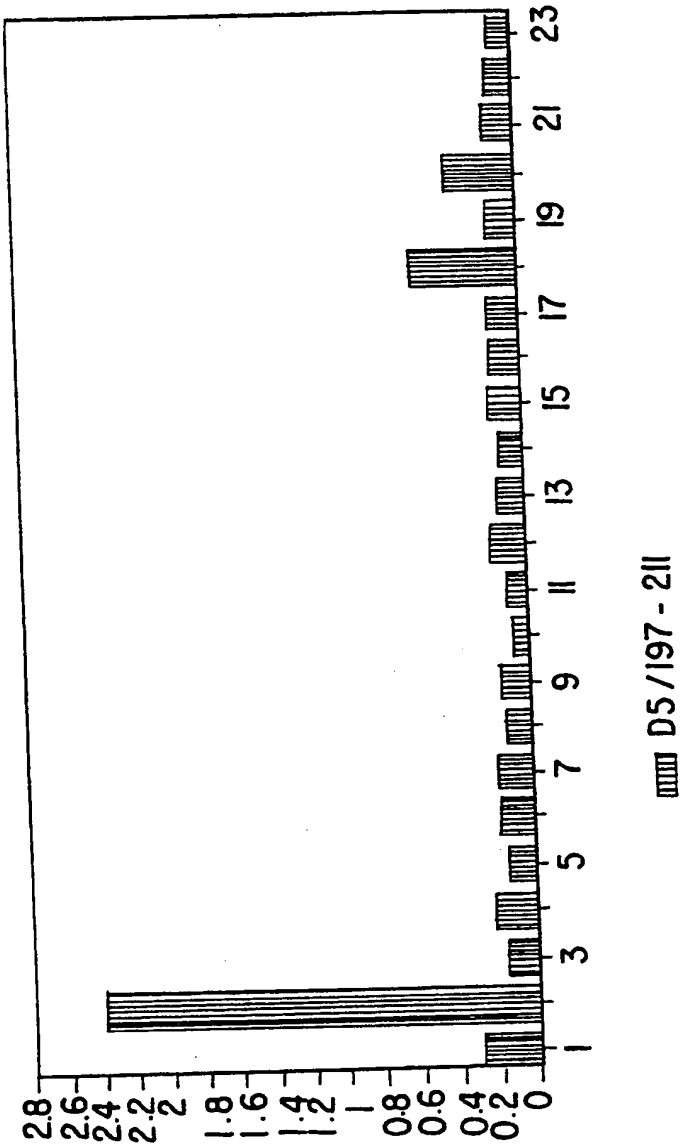

2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 3 5 7 9 11 13 15 17 19 21 23
D5/156 184

FIG. 3F.


2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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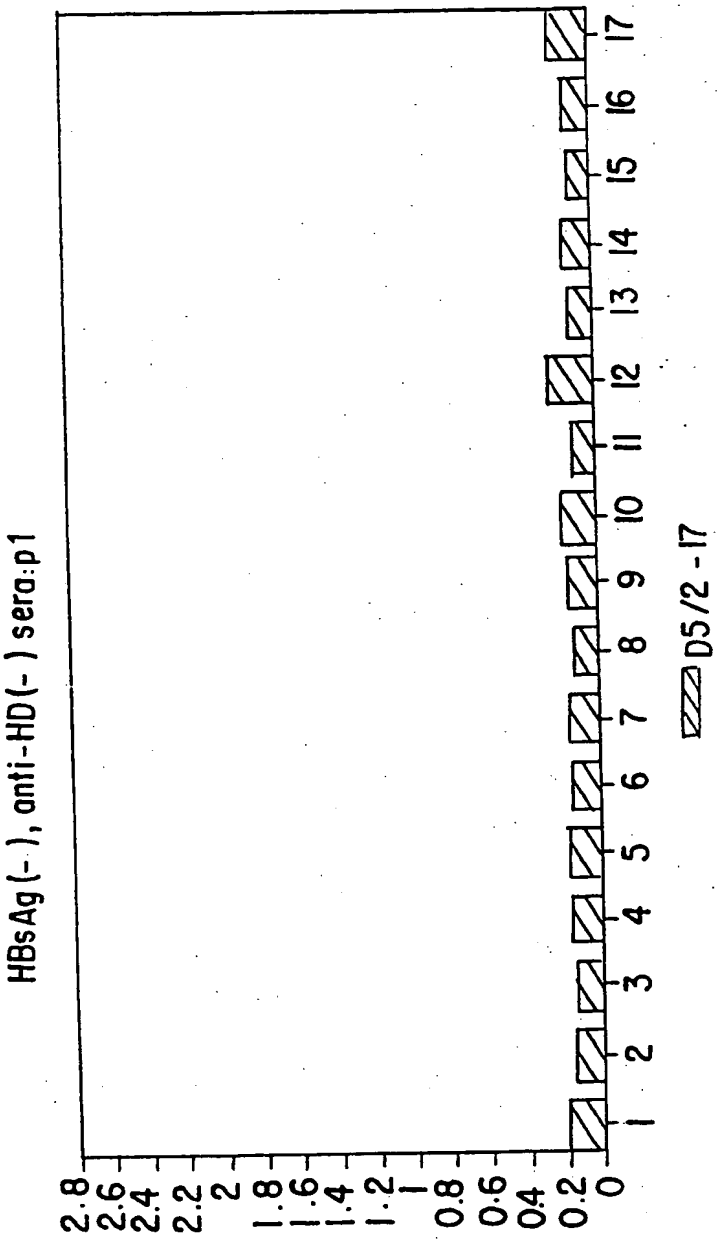
D5/197 - 211

FIG. 3G.


HBsAg (-), anti-HD(-) sera:p1
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
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D5/2 - 17

FIG. 3H.

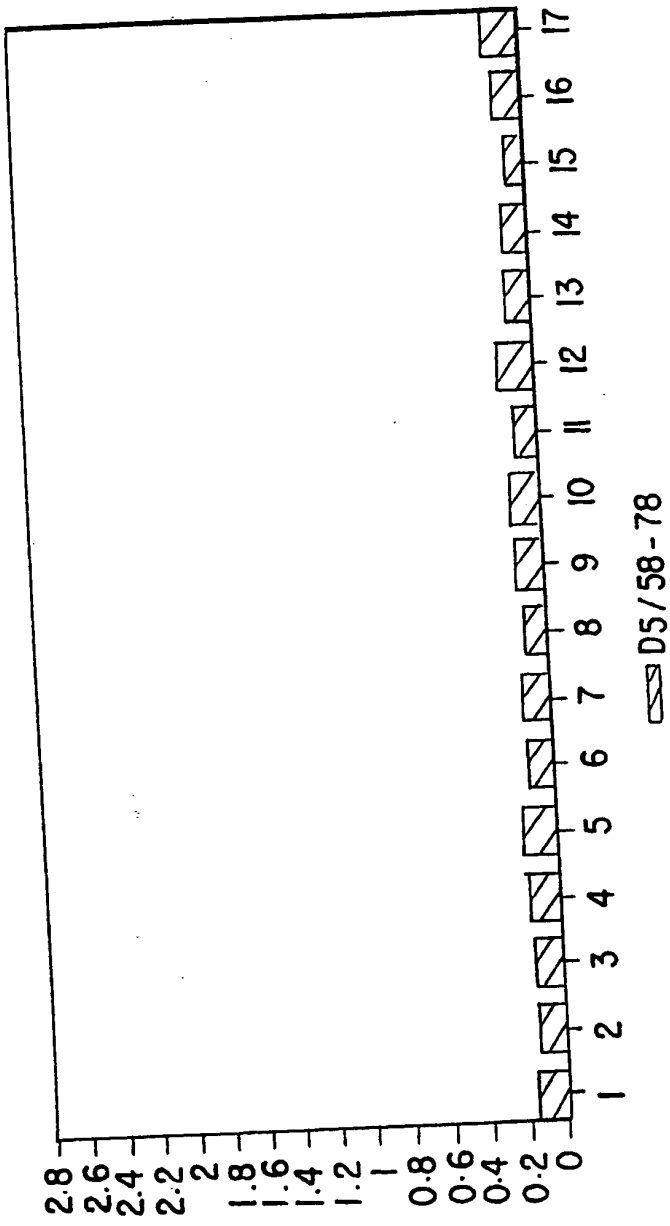
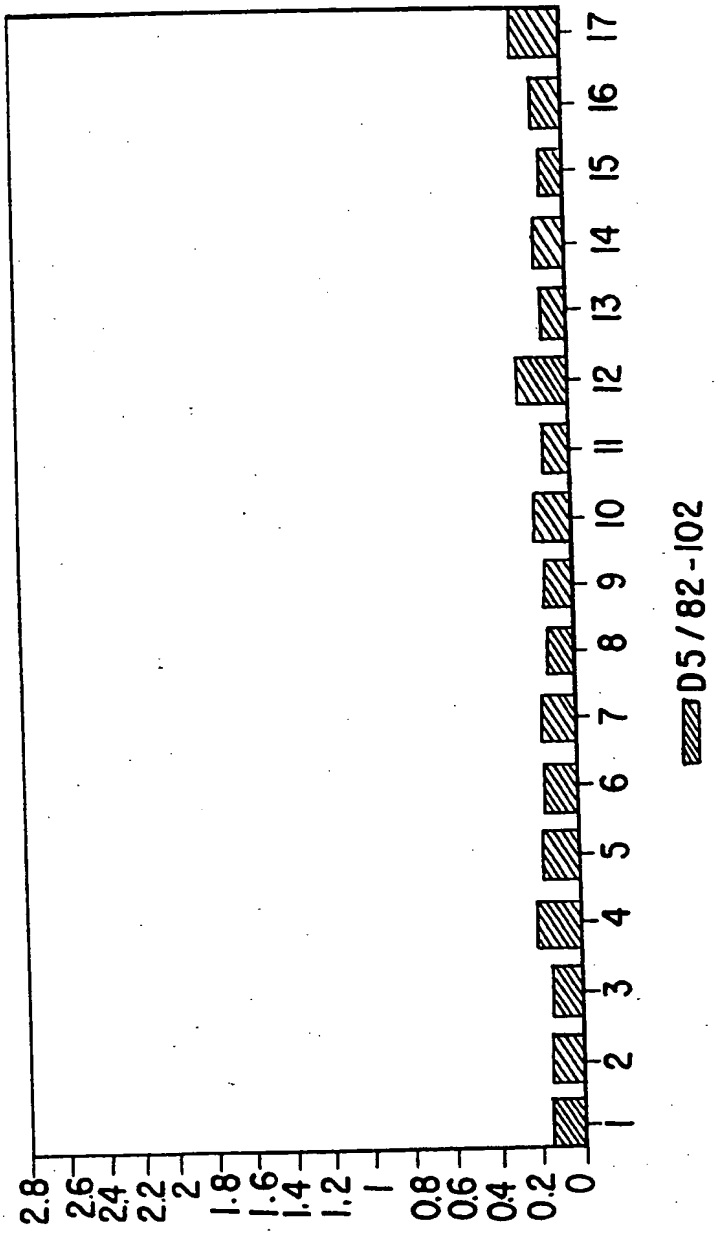

2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
D5/58-78

FIG. 3I.


2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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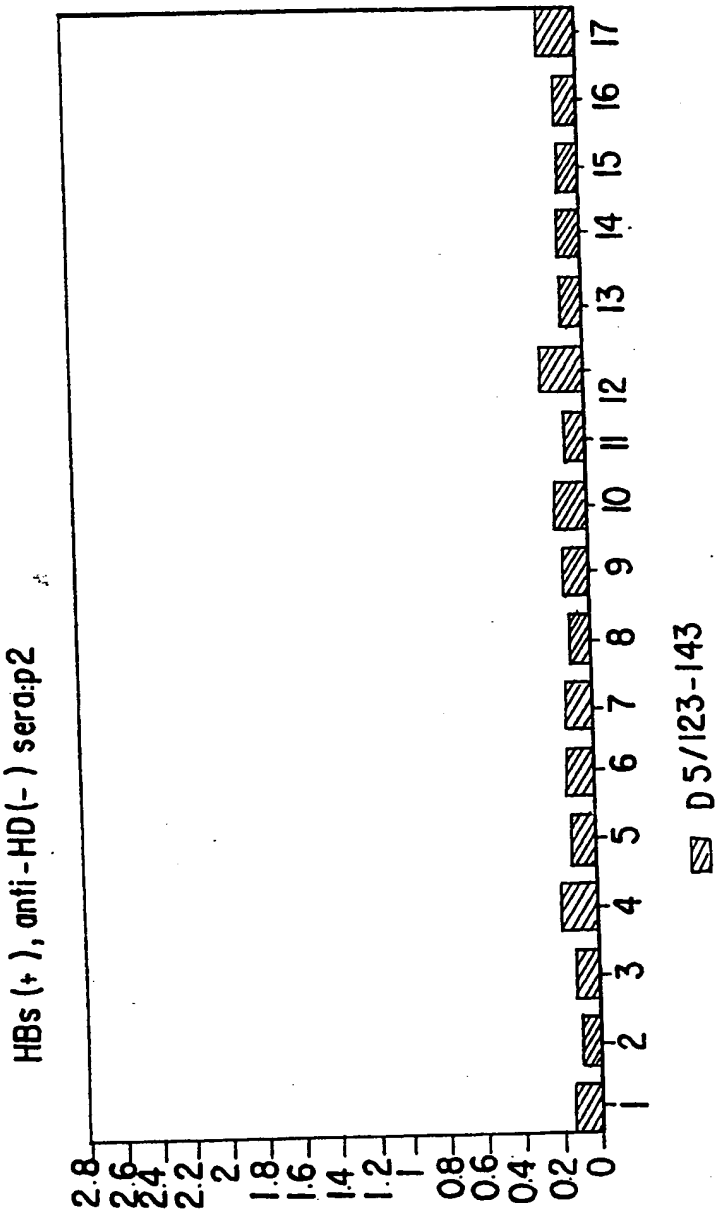
D5/82-102

FIG. 3 J.


HBs (+), anti-HD(-) sera:p2
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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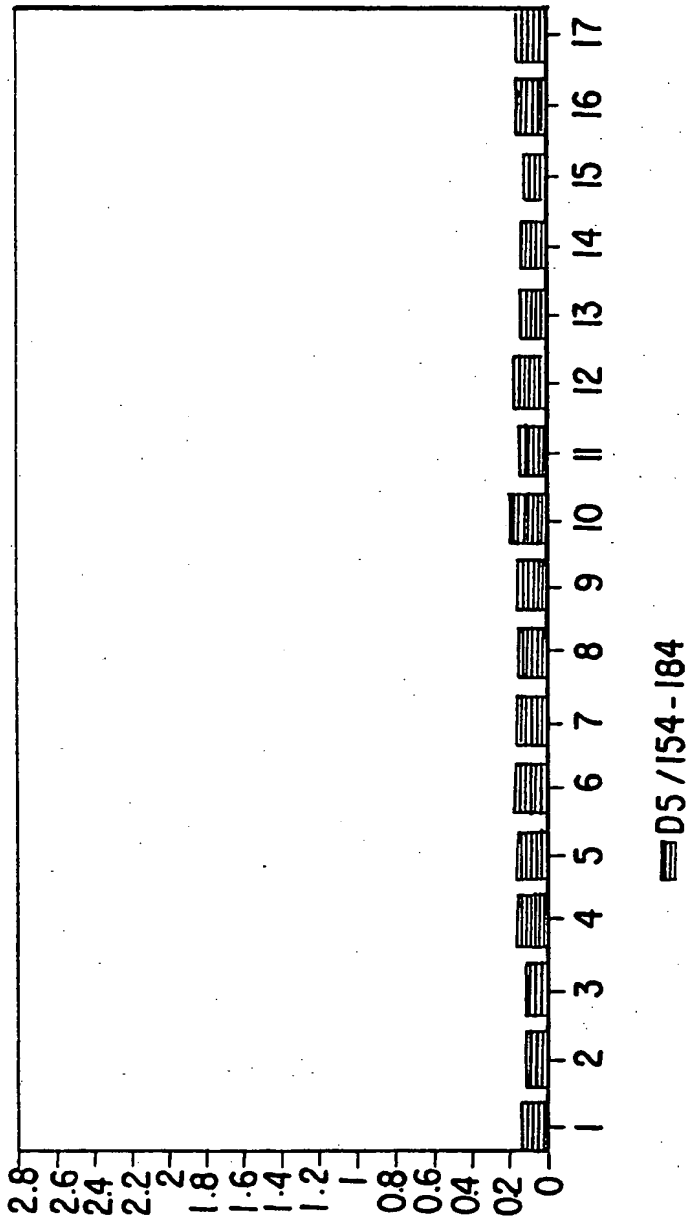
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
D5/123-143

FIG. 3K.


2.8
2.6
2.4
2.2
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1.8
1.6
1.4
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0.8
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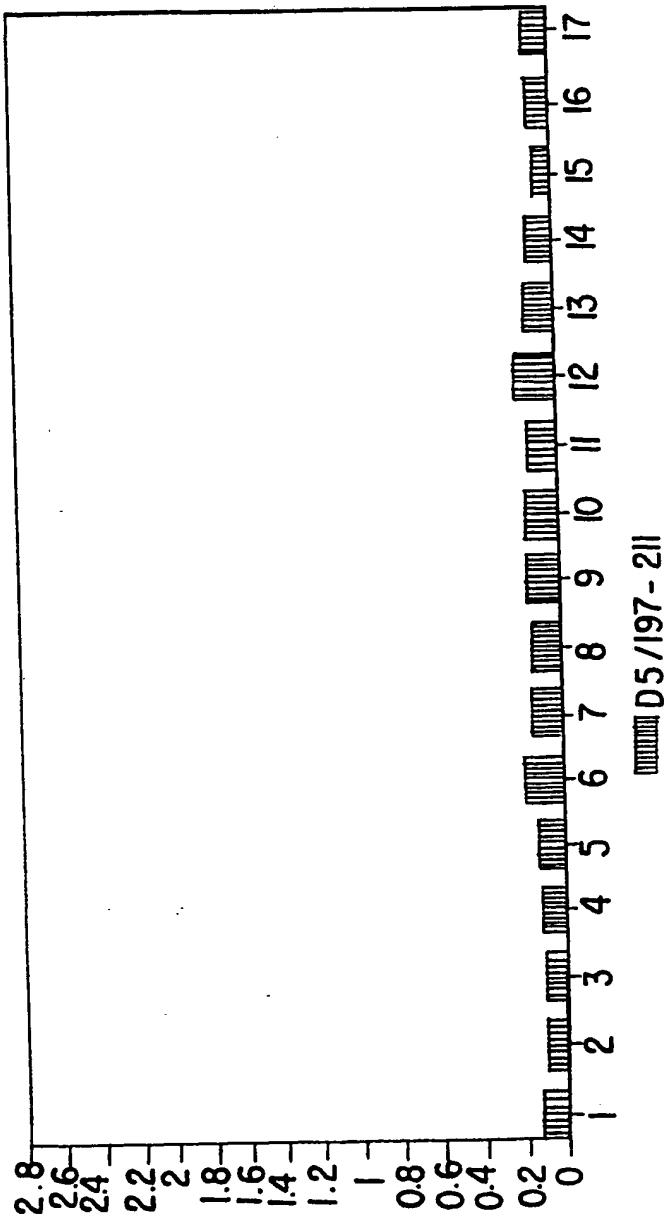
DS/154-184

FIG. 3L.


2.8
2.6
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1.4
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D5/197-211

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06077

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

I.P.C.(5): C07K-7/00; G01N-33/53; A61K-39/00

U.S.: 530/324,325,326,327,328; 435/7; 424/88

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	530/324-328, 435/7, 424/88

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

DIALOG, CAS, SWISSPROT, PIR

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	CABIOS, Vol. 4, NO. 1, issued 1988, B.A. Jameson et al., "The antigenic index: a novel algorithm for predicting antigenic determinants", pages 181-186, see entire document.	1-8,11-12
Y	Proc. Natl. Acad. Sci. USA, Vol. 78, No. 6, issued June 1981, T.P. Hopp et al., "Prediction of protein antigenic determinants from amino acid sequences", pages 3824-3828, see entire document.	1-8,11-12
Y	Nature, Vol. 329, issued 24 September 1987, S. Makino et al., "Molecular cloning and sequencing of a human hepatitis delta (δ) virus RNA", pages 343-346, see especially Fig. 2.	1-8,11-12

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
12 December 1990	28 FEB 1991
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Diane M Offutt for JON P. WEBER

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y, P	Journal of Immunology, Vol. 143, No. 11, issued 01 December 1989, K.F. Bergmann et al., "Hepatitis Delta Antigen Antigenic Structure and Humoral Immune Response", pages 3714-3721, see especially Fig. 1.	1-8,11-12

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. PEPTIDES (1st Product) class 530, subclasses 324-328, (claims 1-8, 11-12).

IMMUNOASSAY (1st Method of use) Class 435, subclass 7, (claims 9, 13).

II. VACCINE (2nd Method of use) Class 424, subclass 88, (claims 10, 14).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.